CANCER RESEARCH

VOL. 12 JUNE 1952 No. 6

CONTENTS

THE OFFICIAL ORGAN OF THE

AMERICAN ASSOCIATION FOR CANCER RESEARCH, INC.

Published by THE UNIVERSITY OF CHICAGO PRESS

CANCER RESEARCH

This journal is sponsored by The American Association for Cancer Research, Inc.; The Anna Fuller Fund; The Jane Coffin Childs Memorial Fund for Medical Research; The Elsa U. Pardee Foundation; and The American Cancer Society.

CANCER RESEARCH is published monthly for Cancer Research, Inc., by the University of Chicago Press, 5750 Ellis Avenue, Chicago 37, Illinois. Subscriptions are by volume only and are payable in advance. The subscription price is $12.50 per volume; the price of single issues is $1.25. Orders for less than a full volume will be charged at the single-issue rate. Postage is prepaid by the publishers on all orders from the United States and its possessions. No extra charge is made for countries in the Pan American Postal Union. Postage is charged extra as follows: For Canada and Newfoundland, 50 cents per volume (total $13.00), 5 cents per issue (total $1.30); for all other countries in the Postal Union, $1.00 per volume (total $13.50), 10 cents per issue (total $1.35).

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For the British Empire, except North America and Australasia:

Cambridge University Press, Bentley House, 200 Euston Road, London, N.W. 1, England. Prices of subscriptions and of single copies may be had on application.

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Claims for missing numbers should be made within the month following the regular month of publication. The publishers expect to supply missing numbers free only when losses have been sustained in transit and when the reserve stock will permit.

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Entered as second-class matter, February 15, 1949, at the post office at Chicago, Ill., under the Act of March 3, 1879.

Acceptance for mailing at special rate of postage provided for in United States Postal Act of October 3, 1917, Section 1103, amended February 28, 1925, authorized June 1, 1950.

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Transfer of the Agent for Mammary Cancer in Mice by the Male*

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In the first crosses between inbred strains of mice with a high and low incidence of spontaneous mammary cancer, a high incidence was noted in the hybrids with maternal parents from the high cancerous stocks, while in the reciprocal hybrids, with paternal parents from these same cancerous stocks, a low incidence was observed (37, 39). These results indicated that a 'maternal influence' was of prime importance in the genesis of this disease in mice; further studies demonstrated that it was transferred in the mother's milk (12), and it will be designated in this report as the mammary tumor milk agent, or agent. The agent has been found to have virus-like characteristics (11, 17, 18, 25, 26, 43).

Subsequent studies on the foster nursing of hybrids born to females of inbred strains with either a high or low incidence of mammary cancer showed that the progeny with mothers of low cancerous strains but nursed by females of the cancerous stocks had the same incidence as did the reciprocal hybrids born to, and nursed by, females of the same cancerous strain (16, 18, 22). Conversely, the reciprocal foster nursing resulted in a low incidence in the hybrids, regardless of the maternal stock. This and other experiments indicated that cytoplasmic and intra-uterine factors were of no real significance, as had been suggested (32, 41). In another cross between mice of two cancerous strains, data were obtained which showed that the agent from the two inbred strains did not have the same 'activity' in the reciprocal F_1 hybrids, mice with the same genetic constitution (13). These observations have been confirmed and extended in other experiments (26).

The role of hormones in the genesis of mammary cancer in mice had been known for many years (40), and in 1939 (16–18) a theory was suggested that these tumors usually resulted from the action of three 'primary' factors—hormonal stimulation, inherited susceptibility, and the mammary tumor milk agent. However, the few tumors that arose spontaneously in mice of strains with a low incidence could not be explained according to the same hypothesis, since these animals would not possess all the causative factors, especially the agent (16). Whether or not a strain has the inherited susceptibility for spontaneous mammary cancer may be determined only by testing the mice after they have obtained the agent, either by nursing or by the injection of an extract. Furthermore, the incidence in susceptible mice without the agent may be no higher than that in so-called nonsusceptible animals, and some considered to be resistant may show a high incidence as soon as they obtain the agent (reviewed in [25]).

In 1944 (31, 34) it was reported that the development of mammary cancer in virgin females was also dependent on an inherited hormonal mechanism, termed the "inherited hormonal influence" (24), acting with the inherited susceptibility and the agent. It was soon demonstrated (29, 30) that the same genes do not control the inherited susceptibility for spontaneous mammary cancer and the inherited hormonal influence, and, in another

* Assisted by grants from the American Cancer Society upon recommendation by the Committee on Growth of the National Research Council; the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service; and the Graduate School Cancer Research Fund of the University of Minnesota.

Received for publication September 6, 1951.

cross, animals of one strain with a 'low susceptibility' for mammary cancer were found to transmit the inherited hormonal influence (26, 36). Other hormonal mechanisms may be inherited, and one has been found that may delay the time of appearance of mammary cancer in virgins and also in breeders (27 and unpublished data).

Heston and his associates (35) have published recently that, in females of the Andervont line of the C3H stock without the milk agent, 38 per cent of the breeders will develop mammary cancer, whereas a low incidence was noted in nonbreeders. In another subline of the same stock without the agent, maintained by Andervont (7), the incidence in breeders was 3 per cent, whereas in mice of our fostered C3H stock less than 1 per cent of the breeders died with mammary cancer (21, 24, and below).

During the past few years several investigators have found a relatively high incidence in the progeny obtained by mating males with the agent to females without the agent of either susceptible (3, 7–9, 21, 28) or relatively nonsusceptible (33, 45) stocks. These results have been interpreted as due to the transfer of the agent by the male (6, 28, 33), since it may be recovered from the contents of the seminal vesicles (7) and the cauda epididymidis (42) of males of cancer stocks.

Further data are presented in this report on crosses between females of either susceptible or comparatively resistant strains and males that possessed the agent. The incidences of mammary cancer will be given for both the females and their progeny, as well as the results obtained for assays of the spontaneous tumors for the agent. In other instances, the agent was recovered from mammary tumors of mice born of parents neither of which had the agent.

MATERIALS AND METHODS

Numerous inbred strains of mice were used in the various crosses, and reference will be made to each when they are discussed.

To prepare extracts of the tumors for assay for the agent, the tumors were ground with sand, extracted with either distilled water or saline (Tables 7 and 11) to make a 10 per cent suspension. This was centrifuged (clinical centrifuge, approximate speed 2,500 r.p.m.) for 5–15 minutes. With the shorter periods, the supernatant fluid was removed and recentrifuged for the same length of time. The final supernatant fluid was diluted to the concentrations given in the tables, and each mouse received 1 cc. of the extract intraperitoneally. The age of the test animals (ZBC mice) at the time of injection was from 23 to 30 days.

EXPERIMENTAL RESULTS

Animals of the C, Bagg albino, strain were obtained from Dr. G. D. Snell in 1942, and since that time 87 breeding females have been observed, and all remained free of mammary tumors. Of the total, 13 per cent were permitted to live for longer than 700 days and 33 per cent for more than 600 days. The average age for the entire group was 561 days.

In the first study on the transmission of the agent by males, eight females of the C strain were mated to two males of the C3H cancerous stock (44), hereafter designated the "Z" strain for simplification. Three of four from one litter and one of four from another litter developed spontaneous mammary cancer. The tumors appeared when the mice averaged 541 days of age, while the four noncancerous mice lived an average of 686 days. Eight to ten litters were born to the cancerous females, while the noncancerous mice had either ten or eleven litters (Table 1).

The CZF_1 (C♀ × Z♂) hybrids were kept either as breeders or virgins, as tabulated in Table 1. There was a total incidence of 45 per cent in 42 hybrids with cancerous C mothers: 64 per cent in virgins and 36 per cent in breeders. When they are listed according to the litter in which they were born, those cast in the first five litters had an incidence of 8 per cent (virgins, 0 per cent; breeders, 10 per cent). All the CZF_1 females born to cancerous C females from their seventh to tenth litters that were continued eventually had mammary cancer, whether they were kept as breeders or as virgins (five per box). The tumors developed earlier in the breeders owing to the increased hormonal stimulation of the mammary tissues. A larger proportion of the nonbreeders were members of the later litters, and this accounts for the higher incidence mentioned above.

Four tumors, diagnosed[1] as undifferentiated carcinomas of the mammary gland, two from C females and two from CZF_1 hybrids (noted in Table 1), were assayed for the milk agent in ZBC susceptible mice without the agent (see Table 9 for incidence in controls). The results for the biological assays are given in Table 2. That the spontaneous mammary tumors from the two females of the C stock had the agent may be seen by the high incidence in the injected animals. The relative concentration of the agent in the extract, based upon the wet weight of tissue, had little influence on the final results. The animals that received the fractions from the CZF_1 tumor which appeared when the mouse was 127 days of age showed the highest incidence and had the earliest average cancer ages.

In another experiment, litter-mate controls from four other litters of the C stock, and born within a period of 10 days, were mated to either Z (with the agent) or Zb (without the agent) males. The five C females bred with males of the Zb line either are living after 25 months or have died noncancerous, and none of their $CZbF_1$ progeny have had mammary tumors. As seen in Table 3, consecutive litters were continued whenever it was possible to do so.

When the male was a member of the cancer Z line, three of the C females developed mammary cancer while two did not. All these C females have had cancerous CZF_1 progeny, as designated in Table 3.[2] Among the offspring of one female, tumors did not appear in any of the progeny born before the eighth litter.

In another series, 29 females of the C stock were mated with either Z or A males with the agent, and, in most instances, litter-mate controls were used. The first five litters, in some cases the first ten litters, born to all the females were killed shortly after birth. Three of the 29 C females are living at 20 months

[1] The author is indebted to Dr. R. A. Huseby for the diagnosis of the tumors.

[2] In these litters, 53 or 54 hybrids that died had mammary cancer. In depleted litters the average cancer age was 239 days.

of age, and fourteen have had mammary tumors—64 per cent of those mated with Z males and 33 per cent mated with A males (Table 4). Not only was there a lower incidence in the latter group, but the females were older when their tumors appeared.

Twenty-seven females of the C stock were mated with cancer Z males in the three experiments, and, of these, sixteen—59 per cent—developed mammary tumors. The progeny of three of the noncancerous females had tumors, indicating that even these females transferred the agent.

Animals of the fostered A strain, called the Ax line, are descended from one of the first litters to be used in the study that demonstrated the mammary tumor milk agent (12). The litter had four females born on 1/8/34 to female No. 35904, a member of the 40th inbred generation of the cancerous A strain (4), with an ancestry of sixteen successive generations of mammary cancer. The young were removed shortly after birth and were fostered by a female of the CBA strain, No. 33562.

The observations for females of this fostered litter were:

No. 38430: died noncancerous but had progeny and descendants with a low incidence.

No. 38431: developed mammary cancer, as did some of her descendants.

No. 38432: had primary lung cancer when autopsied at 563 days of age. Her progeny and descendants have had a low incidence of mammary cancer, with one exception (19), and they are being continued as the Ax line at the present time.

No. 38433: died without mammary cancer but had cancerous offspring.

The pedigrees of the fostered litters have been reported in detail (14, 15), and it was demonstrated that, when mice of one litter are fostered, some may obtain the agent while others may not and that 'infected' females may die noncancerous, although they may have the agent, as may be determined by the appearance of cancer in their progeny. The progeny test must be done before these facts become evident.

From 1943 to 1948 the incidence of spontaneous mammary tumors in breeding females of the Ax line was 0.3 per cent. They were representatives of the 18th–20th to the 33d–35th inbred generations, but only the original female of the line had been fostered. During that time, 688 mice were observed, and only animals that survived for longer than 300 days are included (Table 9). Many of the females were killed after they had attained an age of approximately 15 months because of space limitations. Although the average age of the noncancerous mice was 470 days, a few more animals might have developed mammary tumors had they been permitted to live out their life span. One of the mammary tumors contained extensive areas of squamous metaplasia.

Spontaneous pulmonary tumors are common in mice of the

TABLE 1

OBSERVATIONS ON THE DEVELOPMENT OF MAMMARY CANCER IN FEMALES OF THE C STOCK WHEN MATED WITH Z (C3H) MALES AND THEIR CZF_1 PROGENY

Tabulated According to Litters

C FEMALES			1ST–5TH LITTERS				7TH–10TH LITTERS			
No. mouse	Age cancer	No. LITTERS	Virgins No.	Ca.	Breeders No.	Ca.	Virgins No.	Ca.	Breeders No.	Ca.
124819	+500*	8	0		5	1	4	4	0	
124822	+495*	9	3	0	3	0	0		4	4*
124823	+684	10	0		9	0	2	2	4	4
124828	+496	9	2	0	3	1	3	3	0	
Total			5		20		9		8	
Per cent ca.			0		10		100		100	
Av. ca. age					652		349		215	
Av. nonca. age			631		656					
No. mouse	Age death									
124820	−592	10	6	0	7	0	0		0	
124825	−723	10	0		7	0	0		4	0
124826	−658	11	0		8	0	2	2*	0	
124827	−772	10	0		7	1	3	0	0	
Total			6		29		5		4	
Per cent ca.			0		3		40		0	
Av. ca. age					487		514			
Av. nonca. age			713		629		797		788	

* Four tumors tested for the agent (see Table 2).

TABLE 2

ASSAY OF C AND CZF_1 TUMORS FOR THE AGENT (SEE TABLE 1)

Test animals were ZBC susceptible mice without the agent. Living animals were counted as noncancerous in determining the incidences. The CZF_1 +432 tumor developed in an 8th litter hybrid born to a C female which died noncancerous at 658 days of age.

TUMOR	GM. EQUIV. INJECTED	No.	PER CENT CANCER	AV. AGE Cancer (days)	AV. AGE Nonca. (days)
C +500	2×10^{-2}	31	65	308	414
C +500	10^{-3}	35	74	342	468
C +495[1]	2×10^{-2}	33	70	319	430
C +495[1]	10^{-3}	54	72	344	432
CZF_1 +127*	10^{-1}	27	85	295	429
CZF_1 +127*	10^{-2}	30	93	316	1 living
CZF_1 +127*	10^{-4}	41	90	324	395
CZF_1 +432	2×10^{-2}	38	74	400	2 living
CZF_1 +432	10^{-3}	38	79	337	3 living

* Progeny of [1].

Ax line, but to obtain accurate details for this type of cancer the mice must be continued for several months longer, as the average age of lung tumor incidence may be 19 months (15). Three salivary gland tumors were observed.

Female No. 62327, a member of the eighth generation of the fostered Ax line, developed a mammary tumor when she was 241 days of age. The line had been free of mammary tumors since fostering; the average age at death for females in the direct ancestry was 529 days. Three of the progeny had been mated, and they gave rise to the so-called AxT line that was continued for ten generations (Table 5). The incidence of mammary cancer in this line was found to be 96 per cent, comparable to that seen in breeders of the cancer A stock for the period (19, 22).

That the mice of the AxT line possessed the milk agent, although they were descended from matings between animals without the agent, was shown in a preliminary report of these data (19). This was the first evidence for such an appearance of the agent, and the exact interpretation of the results may not be given even at this time. That this may occur in other isolated instances will be referred to below.

A litter born in 1948 to a female of the 32d generation of the Ax line, No. 136383, was nursed by a lactating female of the cancer A line, No. 136303. There had not been any mammary cancer in the direct ancestry of the Ax female since the line was fostered in 1934. The three females of the "refostered" litter, called the Axa line, developed mammary cancer. Included in the first three generations of the Axa group were 62 breeders, of which 81 per cent had mammary cancer at an average age of 383 days. Five of the twelve noncancerous animals had only one or two litters; in the A stock an adequate hormonal stimulation is required before a high incidence may be expected (16). The results observed among the Axa breeders compare with those seen in females of the cancer A strain maintained during the same period (Table 9).

Sixteen Ax females were used to produce $AxAF_1$ hybrids by mating them with males of the cancer A stock. Nine of the ten examined had primary lung tumors, and all sixteen Ax females died without mammary cancer at an average age of 557 days. The F_1 and F_2 hybrids were continued as breeders, and only one of the second generation developed a mammary tumor (Table 6). In this cross there was no evidence for the transmission of the agent by males of the cancer A stock to females of the same susceptible strain without the agent.

In another study 23 females of the Ax line were mated with males of the cancer Z stock. Twenty of the group survived for more than 500 days (average age, 537 days), while the other three died between the ages of 401 and 435 days. As in the previous group, no mammary tumors were noticed. High incidences, 94 per cent (Table 9) and 66 per cent, are found among breeders and virgins of the Z strain, respectively (26).

The mice of the fostered Z stock, or Zb line, were descended from three females, born 5/27/38, and nursed by a female of the B stock (21). They were of the 44th inbred generation, and, with the exception of one mouse, mammary cancer had been observed in every generation. Representatives of the 9th–27th generations of the Zb line, continued without further fostering and by brother-to-sister matings, were observed during the

TABLE 3

PRELIMINARY DATA ON THE OBSERVATION OF MAMMARY CANCER IN HYBRIDS

Produced by mating females of the C stock with males of the Z (C3H) strain; Z♂ with the agent and Zb♂ without the agent. Mated hybrids are listed according to the litter in which they were born and cancerous hybrids by the litter first to have tumors

C females		Mated	Hybrids	No. litters	Hybrids mated by litters	First litter in which mammary cancer appeared in hybrids
154976	+520*	Z♂	CZF_1	8	1 to 6	3d
154979	+493	Z♂	CZF_1	7	1, 3 to 6	4th
154980	+662	Z♂	CZF_1	9	1 to 6, 8	3d
154984	−664	Z♂	CZF_1	9	2 to 9	8th
154987	−402	Z♂	CZF_1	7	1, 2, 4 to 6	4th
154977		Zb♂	$CZbF_1$	10	1 to 3, 6 to 8, 10	none
154981	−669	Zb♂	$CZbF_1$	9	1, 2, 5 to 8	none
154982		Zb♂	$CZbF_1$	10	1 to 9	none
154985		Zb♂	$CZbF_1$	9	1, 2, 5 to 8	none
154988	−629	Zb♂	$CZbF_1$	10	1, 2, 4 to 9	none

* + = age appearance mammary cancer; − = age at death of noncancerous mouse.

TABLE 4

COMPARATIVE DATA OBTAINED BY MATING FEMALES OF THE C STOCK WITH MALES OF EITHER THE CANCEROUS Z OR A STRAIN

	C females mated to Z males			C females mated to A males		
	No.	Av. age (days)	Av. no. litters	No.	Av. age (days)	Av. no. litters
Cancerous	9	483	9.9	5	554	10.4
Noncancer	3	593	9.7	9	573	9.1
Living	2		9.5	1		10.0
Total	14		9.7	15		9.6
Per cent cancer	64			33		

TABLE 5

DEVELOPMENT OF THE AxT CANCEROUS STOCK FROM AN 8TH GENERATION FEMALE OF THE FOSTERED Ax LINE

Generation	No.	Per cent cancer	Av. ca. age
F_8	1	100	241
F_9	3	100	327
F_{10}	11	100	279
F_{11}	34	97	302
F_{12}	35	92	283
F_{13}	61	92	305
F_{14}	66	100	278
F_{15}	39	95	289
F_{16}	6	100	316
F_{17}	3	100	331
Total	259	96	292

period of 1943 and 1948, and, among the 800 breeders, 3 or 0.4 per cent died with mammary cancer (Table 9). One of the tumors had extensive areas of squamous metaplasia.

One adenocarcinoma from a mouse of the seventeenth generation of the Zb line was tested for the milk agent (26). Although the line had been free of mammary cancer, observations

TABLE 6

INCIDENCE OF MAMMARY CANCER IN THE HYBRIDS RESULTING FROM RECIPROCAL MATINGS BETWEEN MICE OF THE A AND AX LINES

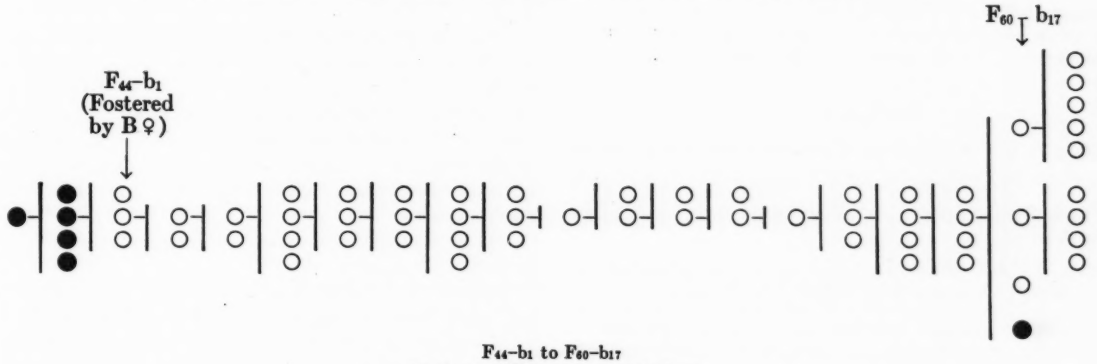
MATINGS	HYBRIDS	No.	PER CENT CANCER	AV. AGE cancer (days)	AV. AGE nonca. (days)
A♀ × Ax♂	$AAxF_1$	117	94	276	266
F_1♀ × F_1♂	$AAxF_2$	133	96	285	289
F_2♀ × F_2♂	$AAxF_3$	149	97	264	373
Ax♀ × A♂	$AxAF_1$	107	0		549
F_1♀ × F_1♂	$AxAF_2$	152	1	346	553

females had cancerous $ZbZF_1$ progeny, as did the one cancerous mouse. In Table 8 the hybrids are listed according to whether or not the Zb females became "infected" with the agent from the Z males. The 23 noninfected Zb females had 352 progeny and descendants of the $ZbZF_1$–F_3 generations; none had mammary cancer, and the average age at death was 487 days. Before infection six Zb females had seventeen F_1 progeny and 43 other descendants that lived to an average age of 478 days without developing cancer; after infection 88 per cent of their 40 F_1 and F_2 hybrids had mammary cancer. The average cancer age was 270 days, while the five noncancerous mice lived to an average of 507 days. Only one of the cancerous $ZbZF_1$ mice was born in the second litter; the others were members of the third and following litters born to the Zb females.

Four $ZbZF_1$ young were born to the cancerous Zb female, No. 71171, in her second litter; they and their nineteen F_2 and F_3 descendants lived to an average age of 462 days, and mammary cancer was not observed in any mouse. Two F_1 females born in the third litter to the same Zb female and their four

TABLE 7

PEDIGREE OF PART OF THE ZB LINE OF THE C3H STOCK AND BIOLOGICAL ASSAY FOR THE MILK AGENT OF MAMMARY TUMOR FROM A MOUSE OF THE 17TH GENERATION



F_{44}–b_1 to F_{60}–b_{17}
56 nonca. (○): av. age, 506 days
1 cancer (●): av. age, 508 days

GM. EQUIV. INJECTED	AGE WHEN INJECTED (DAYS)	No.	PER CENT CANCER	AV. AGE cancer (days)	AV. AGE nonca. (days)
2×10^{-2}	22–23	30	90	368	354
10^{-3}	22–23	33	82	387	556

among the injected animals, ZBC hybrids, revealed that the tumor carried the milk agent (Table 7). None of the progeny of the cancerous Zb female was continued, but three littermates of the cancerous animal and ten of their progeny remained noncancerous, with an average age at death of 491 days.

After females of the cancer Z line were mated with males of the fostered Zb line, 94 per cent of the resulting $ZZbF_1$ hybrids and their progeny ($ZZbF_2$) were found to have mammary cancer when they were used as breeders (Table 8 [21]).

Thirty Zb females were used to produce $ZbZF_1$ hybrids by crossing them with males of the Z line with the agent, and hybrids of the F_1–F_3 generations were observed, also as breeders. The data previously published (21) for these mice are retabulated in view of the findings that the males may transmit the agent, because in the earlier report it was shown that some of the descendants of some Zb females had mammary cancer.

One of the Zb females (3 per cent of the total) mated with Z males had a mammary tumor when 416 days of age, and the others died at an average age of 528 days; 25 of the 29 noncancerous mice survived for more than 584 days. Six of these Zb F_2 progeny all had cancer at an average age of 217 days.

In this cross between Zb females and Z males several of the females became infected with the agent from the male, although only one had a mammary tumor. The progeny born to these females before they became infected remained noncancerous, but after infection the offspring born to the same females had a high incidence, as did their offspring.

ZBC females have been used to assay normal and cancerous tissues for the presence of the mammary tumor milk agent (24, 26). These mice are produced by reciprocal matings between animals of the Ax and Zb lines, and the resulting F_1 females, called $AxZbF_1$ (Ax♀ × Zb♂) or $ZbAxF_1$ (Zb♀ × Ax♂), are then mated with Zb males to produce the ZBC test animals, although some were of the next generation or ZBC_2 hybrids (ZBC♀ × Zb♂).

At one period, the supply of Zb males was not adequate for the production of the experimental mice, and the F_1 females were mated with Z males that had the agent. In Table 9 the incidences of mammary cancer in the various groups have been tabulated according to the presence or absence of the agent in the males. Again, only noncancerous mice that lived longer

than 10 months are included. In determining the incidences, cancerous mice only of the first generation of any group were counted. As soon as cancer was noted in any mouse, their littermates and descendants were removed from the breeding colony but were continued in another series to obtain these data.

In matings where males of the Z line were used, an indication of "infection" with the agent when transferred by the males may not be obtained from these figures. The number of infected females may be secured only by observing all the progeny of the females being tested, but for obvious reasons this cannot be done without sacrifice to other experiments. As has been noted, a low incidence is usually observed in the offspring born to females prior to infection, but after this occurs the progeny born to the same mother have a high incidence. The mother may or may not develop mammary cancer.

In other cases, the females were mated with Zb males, and the results demonstrate that either the females or their progeny obtained the agent from some source, as may be shown by either the progeny test or biological assay of the tumors.

One mammary tumor, a papillary cystadenoma, was observed among the $AxZbF_1$ and $ZbAxF_1$ females after they had been mated with males of the Zb line. The tumor was recorded when the animal was 548 days of age, and none of the progeny born to the cancerous female, No. 88071, was continued. The incidence for the group was 0.1 per cent, and the noncancerous animals survived to an average age of 499 days (Table 9).

Twenty-five ZBC females developed mammary cancer when they were mated with Zb males and were descended from mothers that also had been bred with males of the same subline. The incidence for the group was 1.8 per cent (Table 9), and the average ages were: cancer, 424 days; and noncancer, 525 days.

The cancerous ZBC females were members of twenty separate litters, but nine mice, with an average age of 349 days, belonged to four litters consisting of a total of twelve mice. Included in the other sixteen litters were 43 mice, of which sixteen, or 37 per cent, had tumors (average age, 467 days). Two of the mothers of cancerous animals in this series had eight progeny born in succeeding litters, and none developed mammary cancer.

Progeny of the cancerous ZBC females were continued only in isolated instances.

Female No. 124036 had cancer when she was 498 days of age, gave birth to nine litters, and the six young born in her third and seventh litters died noncancerous. Two litter-mates of the cancerous female and their eight progeny also remained free of tumors. In another litter, two of four females had cancer at 593 and 347 days, while the three progeny born to the former in her second litter did not. The noncancerous litter-mates survived for 740 days.

Litter-mates Nos. 115646 (+266) and 115647 (+258) and three females born to the former in her second litter all had cancer at an average age of 282 days. The mother of these cancerous females was mated to a Zb male and maintained until she was 567 days of age and had eight litters.

The mother of the cancerous ZBC females, Nos. 121074

TABLE 8

MAMMARY CANCER IN THE HYBRIDS PRODUCED BY RECIPROCAL MATINGS BETWEEN ANIMALS OF THE Z AND ZB LINES

F_1 mice were mated *inter se* to give F_2 hybrids, etc.

MATINGS	No.	PER CENT CANCER	No.	PER CENT CANCER	No.	PER CENT CANCER
Z ♀ × Zb ♂	$ZZbF_1$		$ZZbF_2$		$ZZbF_3$	
	138	94	136	94	0	
Zb ♀ × Z ♂	$ZbZF_1$		$ZbZF_2$		$ZbZF_3$	
23 "noninfected" Zb ♀	104	0	160	0	88	0
6 Zb ♀:						
before infection	17	0	30	0	23	0
after infection	18	78	22	95	0	
1 Ca. Zb ♀:						
before infection	4	0	13	0	6	0
after infection	2	100	4	100	0	

TABLE 9

OBSERVATION ON MAMMARY CANCER OBTAINED FROM 1943–48 IN BREEDERS OF THE A AND Z (C3H) STOCKS, WITH (A AND Z) AND WITHOUT (AX AND AB) THE MILK AGENT

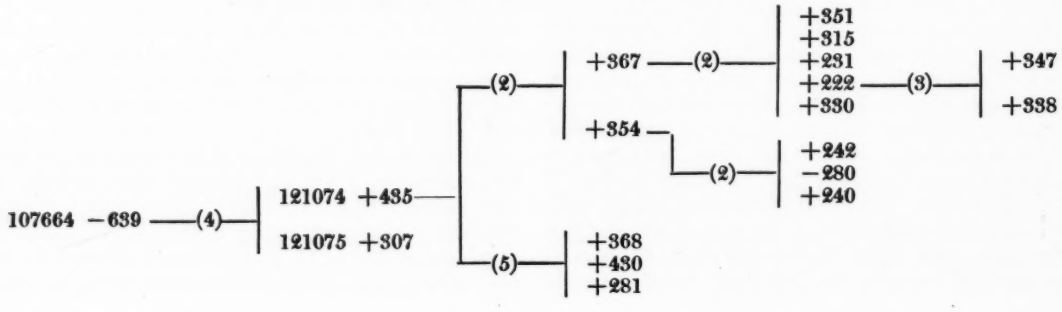
The F_1 and ZBC hybrids without the agent are tabulated according to matings with either Z or Zb males

	No.	PER CENT CANCER	AV. AGE cancer (days)	AV. AGE nonca. (days)
Z (C3H) stock	524	94	282	325
A stock	384	82	344	394
ZAF_1 (Z ♀ × A ♂)	69	97	299	361
AZF_1 (A ♀ × Z ♂)	85	97	320	414
Zb stock	700	0.4	482	475
Ax stock	688	0.3	441	470
$ZbAxF_1$ × Zb ♂ or	998	0.1	548	499
$AxZbF_1$ × Z ♂	370	1.1	527	506
ZBC × Zb ♂	1,423	1.8	425	525
ZBC × Z ♂	123	2.4	412	524

TABLE 10

THE APPEARANCE OF MAMMARY CANCER IN THE PROGENY AND DESCENDANTS OF FEMALE NO. 107664

Cancer or noncancer ages denoted by + or −; parentheses in line to progeny denote litter in which they were born. All matings were with Zb males



(+435) and 121075 (+307), lived for 639 days, and her cancerous progeny were born in her fourth litter. All matings were with Zb males. The five progeny born to No. 121074 in her second and fifth litters and nine of their ten descendants also developed mammary cancer. Thus, 93 per cent of the progeny and descendants of No. 121074 died cancerous (cancer age, 315 days, and noncancer age, 280 days). The pedigree for this family is given in Table 10.

Although none of the tumors from animals of these later two groups was tested for the agent, the incidence and average cancer age, 282 and 315 days, were indicative of the presence of the agent regardless of the fact that all matings were with Zb males.

Males of the Z line with the agent were mated to 370 $ZbAxF_1$ or $AxZbF_1$ females, and four, or 1.1 per cent, had mammary cancer (Table 9). One tumor, an adenocarcinoma, observed in a mouse at 20 months of age, had considerable connective tissue.

Progeny of three of the cancerous females were observed. Eleven progeny of two females, born in each case in their fifth litters, died without cancer. The other cancerous female, No. 118187, developed a tumor when 510 days of age and had ten litters. Five of the six mice born in her fifth litter and thirteen of fourteen of the next generation had cancer. The two noncancerous animals died at 255 and 258 days, while the average cancer age for the others was 295 days. Thus, all the progeny and descendants of No. 118187, eighteen in number, that survived for longer than 260 days eventually developed mammary cancer. Assays for the agent were not made, but the data indicate that the agent was involved in the etiology of these tumors.

Two per cent of the ZBC females that were mated with Z males developed mammary tumors at an average age of 412 days (Table 9). Progeny of the three cancerous animals were not continued, and the tumors were not assayed for the agent.

In several isolated groups of animals, it was detected that, when F_1 females had been mated with Z males, the F_1 females died noncancerous, but some of their ZBC progeny, mated with Zb males, developed mammary cancer. The results suggest that the F_1 females became infected from the Z males and the agent was transferred in the milk to the progeny. These cancerous ZBC females have not been included in any of the tabulations listed in Table 9.

Five F_1 litter-mates were mated with a Z male, had from six to ten litters, and survived to an average age of 628 days. Four of the progeny born to one female in her fifth litter were continued, and three developed cancer. Tumor No. 7389 from one of the progeny (No. 102395 + 323) was assayed for the agent by injecting 1 cc. of a 1 per cent suspension following centrifugation for 10 minutes. Thirty-three ZBC mice were injected, but only twelve survived to the average cancer age for the group, or 318 days, and of these six had tumors. Thus, it seemed probable that the tumor carried the agent.

A male of the Z strain was mated with three $ZbAxF_1$ females from another litter. They lived to an average of 671 days, and each had from seven to eleven litters. One of the progeny (No. 109425 + 301), born in the second litter to a F_1 female that survived for 726 days, had cancer, as did five of her progeny. The average cancer age for the six cancerous mice was 441 days.

Of five $AxZbF_1$ litter-mates that were crossed with a Z male, one (listed above) had a mammary tumor when 603 days of age, and the others died noncancerous; No. 98109 had seven litters and lived for 533 days. The seven young born in her sixth litter and 28 of 33 of their offspring had cancer. The incidence in the group was 88 per cent; cancer age was 324 days and noncancer age, 459 days. One mammary tumor from a descendant of No. 98109 was tested for the agent, and the high incidence in the injected mice demonstrate the presence of the agent in the tumor (Table 11). The lower incidence in the mice injected with the 10 per cent suspension was probably due to the fact that three of the mice died before they had attained the average cancer age for the group.

Two $ZbAxF_1$ sisters were killed at 569 days of age after they had been mated with a Z male, and each had eight litters. Four mice born to No. 118206 in her fifth litter and two females born

TABLE 11

TUMOR NO. 7576: ORIGIN AND TEST FOR AGENT

The mother of the cancerous mouse was mated to a male (Z) with the agent; other matings were with Zb males

$AxZbF_1$ ♀ × Z♂		Progeny Ca.	Progeny nonca.	Descendants Ca.	Descendants nonca.
98108 +603					
98109 −533	(6)	7*	0	28	5
98110 −524					
98111 −365	(2)	0	4		
98112 −413					

* Tumor no. 7576 (+302 days) tested for agent: Extracts injected into ZBC females.

Gm. equiv. injected	No.	Per cent cancer	Av. age Cancer (days)	Av. age Nonca. (days)
10^{-1}	18	72	367	363
10^{-3}	25	80	430	491
10^{-4}	20	90	397	442

TABLE 12

DEVELOPMENT OF MAMMARY CANCER IN THE PROGENY OF A $ZbZxF_1$ FEMALE MATED WITH A Z MALE AND TESTS SHOWING THE PRESENCE OF THE AGENT

Other matings were with Zb males. Numbers in parentheses following female mouse number, indicate number of litters and number in line to progeny, litter in which mice were born

$ZbAxF_1$ ♀ × Z♂		Progeny Ca.	Progeny Nonca.	Descendants Ca.	Descendants Nonca.
118206 −569 (8)	(5)	0	4		
118207 −569 (8)	(2)	0	2		
	(5)	4*	0	4	0

* Tumor no. 8075 tested for agent.

Gm. equiv. injected	No.	Per cent cancer	Av. age Cancer (days)	Av. age Nonca. (days)
10^{-2}	68	68	335	522
10^{-4}	50	54	329	521

in the second litter to her sister died noncancerous. All the progeny born to No. 118207 in her fifth litter and their progeny had cancer (average age, 234 days); one was tested and found to have the agent. The pedigree chart for the group and the assay data are given in Table 12. Again we note that the mice born in an early litter, in this case the second, failed to develop cancer, whereas the progeny born to the same mother in her fifth litter developed cancer and they had cancerous progeny.

Twelve Zb females were mated to males of the cancerous dilute brown stock, sublines D_1 or D_2 (26), and eight lived longer than 535 days without having mammary cancer. The F_1 hybrids were maintained as virgins and used in other studies before the oldest had attained the age of 12 months. Prior to this time, a mammary tumor appeared in one ZbD_1F_1 female, No. 98320, when she was 264 days of age. The Zb mother survived

for 641 days, had eight litters, and only the one F_1 hybrid had been saved of her eighth litter. The F_1 tumor, No. 7223, was tested and found to have the agent (Table 13).

In earlier studies (18), females of the C57 black (B) stock had been mated with males of either the A or the Z cancer stocks. None of 62 B females mated with A males developed mammary cancer, although the average age at death was 577 days. Of this number, 29 lived for longer than 600 days (average, 677 days). In another series 24 females of the B stock lived for an average of 418 days and failed to have mammary cancer after being mated with males of the Z stock. At the same time, four of eight females of the B strain that had been fostered by

TABLE 13

RESULTS OBTAINED FOLLOWING THE INJECTION OF EXTRACTS OF TUMOR FROM NO. 98320, A ZBD_1F_1 FEMALE ($ZB♀ \times D_1♂$)

Gm. equiv. injected	No.	Per cent cancer	Av. age Cancer	Av. age Nonca.
5×10^{-2}	19	79	315	561
10^{-3}	27	89	372	501

females of the A strain developed mammary tumors (cancer age, 447, and noncancer age, 477 days).

Table 14 presents a summary of observations on the development of mammary cancer in females of various stocks when they were mated with males of two stocks with (A and Z) and two without (Ax and Zb) the agent.

TABLE 14

SUMMARY OF OBSERVATIONS ON THE DEVELOPMENT OF MAMMARY CANCER IN FEMALES WITHOUT THE AGENT WHEN MATED WITH MALES WITH (Z AND A) OR WITHOUT (ZB AND AX) THE AGENT

Stock of females	From table	Mated Z males (per cent ca.)	Mated A males (per cent ca.)	Mated Zb males (per cent ca.)	Mated Ax males (per cent ca.)
C	1, 3, 4	59*†	33*	0*	
Ax	6, 9, text	0	0	0	0.3
Zb	8, 9, text	3†		0.4	0
$ZbAxF_1$ or $AxZbF_1$	9	1†		0.1	
ZBC	9	2†		2	
B	Text	0	0		

* Data not complete.

† Some females died noncancerous but transferred the agent to their progeny.

DISCUSSION

As shown in recent reviews (25, 26), the mice born of mothers with a low incidence of spontaneous mammary cancer and of fathers from strains in which the females have a high incidence usually had the same incidence as their mothers. Some exceptional cases have been found, the first published by Strong (45) in 1943. In that study, females of the low cancer JK strain were crossed with males of the cancerous C3H stock. The incidence was 71 per cent in F_1 hybrids with JK mothers and 83 per cent among those with C3H mothers. The tumors appeared about 2 months earlier in animals of the latter cross. Strong permitted the sexes to remain together, so that the females were nursing one litter while the next was developing *in utero*. It was assumed that the "extensive or excessive use of the female mice" in this particular cross was "an effective way of bringing about spontaneous tumors of the mammary tissues."

In 1945 (3) Andervont reported that, when females of the C strain were mated with males of the cancer C3H stock, 60 per cent of the F_1 females developed mammary tumors. Eleven of the tumors were tested, but all failed to show the presence of the agent. One of the nineteen C females also had mammary cancer, but it did not transfer the agent as shown by foster nursing. Since he had previously noted that when females of the C stock obtain the milk agent a high incidence resulted (2), Andervont stated that the data indicated, but did not prove, that the agent was not involved in the genesis of these tumors. In a succeeding report of the same cross, Andervont (4) observed four hybrids with tumors appearing between 4 and 6 months of age which had the agent, as was demonstrated by the use of these cancerous mice as foster mothers.

The relatively high incidence for the C ♀ × C3H♂ cross was confirmed in other studies (9), but when C3H males without the agent were mated with C females, 19 per cent of their hybrids developed cancer at an average age of 25 months, 3 months later than when males with the agent were employed. An incidence of 3 per cent was observed in breeders of the inbred C3H line without the agent at an average age of 22 months (7).

Andervont and Dunn (9) suggested several possibilities to explain their experimental data, as follows: "Regardless of whether the agent appeared in the hybrids through contamination, or through the activation of a weak agent harbored by the strain C females, or by the transmission of the agent from the C3H males to Strain C females or their F_1 hybrids *in utero*, or whether the agent arose *de novo*, the fact remains that a highly active tumor inciter appeared suddenly in female mice and was transmitted to their descendants."

In discussing these possibilities, the author stated that transmission by contamination seemed improbable, for, if this was a route of infection, such contamination should occur with greater frequency, and mice other than the hybrids derived by mating C females and C3H males should become infected. Suggestive evidence for the transmission of the agent by the males was seen in the comparative incidences among hybrids with fathers with and without the agent. However, the mating of C females to C males after the females had been bred with C3H males without observing an increased incidence of cancer in the C progeny indicated that the C females had not become infected from the C3H males, as they did not transfer the agent (9). That the hybrids might have secured the agent in the milk of their C mothers was tested by Andervont and Dunn by alternate matings with C3H and C males (9), x-radiation (10), and increased hormonal stimulation by the use of stilbestrol pellets (6). Only negative results were obtained in this series of experiments. If the male with the agent could infect the young *in utero* without infecting the mothers, the authors (9) asserted that certain observations in their experiments, as well as those published by other workers, might be explained. Under these circumstances, if the agent remained attenuated either before or after it got into the embryo, this would account for the appearance of tumors in very old mice; if the activity of the agent was enhanced or a variant was produced, the development of tumors in young animals could be explained[3]. Andervont and Dunn (9) also believed the possibility of the agent appearing *de novo* deserved consideration, but only by the elimination of the other theories might such evidence be attained.

In 1949 (33) Foulds published on the cross between females of the C57 black (B) and males of the cancer R_3 strains where an incidence of 15 per cent was seen in the F_1 hybrids. He observed more tumors among mice born in certain litters than should be expected, and animals of several groups were continued for four generations. In certain lines a high incidence of mammary cancer was found, and tests revealed the presence of the agent in some of the spontaneous tumors; in other lines a low incidence of tumors was seen. Few tumors were observed among the hybrids with fathers of the cancerous R_3 stock without the agent. Foulds explained his results in the BR_3 cross as due to the erratic transmission of the agent by males of the cancer stock. This theory has since been accepted by others (6, 28, 42) to account for certain experimental data.

That some mammary tumors appeared in animals without the mammary tumor milk agent was taken into consideration when the theory was advanced to account for the genesis of these tumors in mice (17). Strong (45) associated the increased hormonal stimuli to which the females in his experiment were subjected as the predominating influence in the development of mammary cancer. In the lines of fostered mice derived from the Andervont cancer C3H stock, the incidence among breeders without the agent was reported by Heston *et al.* (35) to be 38 per cent, while Andervont and Dunn (7) found tumors in only 3 per cent. The incidence in the virgins in the former group was 4 per cent (35). Heston and his associates (35) concluded that the genetic constitution of their animals, combined with intensive breeding, was adequate to result in a relatively high incidence in mice without the agent.

In a recent publication Andervont (6) recognized two problems in the genesis of mammary cancer in hybrids of the C and C3H cross. The development of tumors in young animals resulted, in part, from the action of the agent transferred by the male, whereas tumors appeared in old mice because of their genetic constitution and intensive hormonal stimulation.

The sublines of C3H mice, although derived from the same stock about 20 years ago (44), maintained by Andervont and the author, must be considered as separate lines, for tumors from mice of one line will not grow progressively when they are transplanted into animals of the other colony. Andervont (1) stated in 1941 that data obtained with mice of his line could not be controlled by observations secured from mice of other groups. This difference may account for the observations made on the cross between C females and C3H males from the two laboratories.

Although an accurate figure cannot be given, probably less than 5 per cent of the C females had mammary cancer when they were mated with C3H males in the numerous studies made by Andervont. In our series of 27 C females mated with males of our cancer C3H or Z strain, 16 have had mammary tumors, and two of the total are still under observations. It was evident among the progeny of these cancerous mice that were continued that the females transferred the agent. In addition, a few C females that died noncancerous also had the agent, since some of their progeny developed tumors and the agent could be demonstrated by biological assay. When Zb males without the agent were mated with the C females, the

[3] If this were the route of infection, the young born in inbred cancer-susceptible stocks also would become infected *in utero* from their fathers with the agent, in which case foster nursing would be relatively ineffective.

five females and their progeny have remained free of tumors, and, although most of these mice are still living, mammary tumors have been found (Table 3) in the litter-mate controls when they were mated with males with the agent, and some of their progeny have had tumors.

Although the number of mice is not adequate for definite conclusions, in our series an indication was seen that the relative time of development of the tumors in females of the C stock may be directly correlated with the number of litters. In one study the females were kept in the breeding pens, and the young were removed shortly after birth. This method of breeding permitted the females to have more litters within a given period than when the females were removed from the breeding pens during pregnancy and were not returned until after their young had been raised.

Tumors have not appeared in any of the CZF_1 hybrids born prior to the third litter, suggesting the probable time of infection by the male, since, following the injection of the agent into older mice, the agent was transferred to the young born in the first litter after the injection (unpublished data).

According to the most recent publication of Andervont and Dunn (10), tumors appearing before the mice were 15 months of age may have the agent, but they were unable to recover the agent when the mice were older at the time they developed their tumors. The two tumors from C females developed after the mice were over 16 months of age, and they possessed the agent.

From the studies cited above and the observations presented in this report, it now appears to be well established that males of cancer strains of mice may transfer the agent at the time of copulation to infect females of either susceptible or relatively resistant strains and that these infected females may propagate and pass the agent in the milk to their progeny. The infected females of susceptible strains may or may not develop spontaneous mammary cancer.

That there is a considerable variation in the sensitivity of females of various susceptible groups without the agent to become infected with the agent when transferred by males of cancer stocks was shown by the experimental data given above. Females of the fostered A stock (Ax line) did not develop tumors when they were bred with males of the cancer lines of the A and Z strains, and in the $AxAF_1$ hybrids there was no increase in the incidence (20). When the C females were mated with males of the A strain a lower incidence of mammary tumors and a later average tumor age were noted than when males of the Z strain were used.

In a series of 30 Zb females mated with males of the same cancer line, 3 per cent of the females had tumors and 20 per cent became infected but died noncancerous. Mothers of the latter group had cancerous progeny and descendants, and here again the litter in which the progeny was born influenced the results ([21] and Table 8).

Low incidences of tumors were also seen in the F_1 and ZBC hybrids, derived by crossing the Ax and Zb mice, when they were mated with Z males, but several females of these groups became infected. Cancerous progeny and descendants were observed, and biological assays showed the agent to be present in the tumors.

None of the 86 females of the C57 (B) strain had tumors when they were mated with males of either the A or Z strains, but during the same period four of eight B females that obtained the agent by foster nursing developed mammary cancer, showing that mice of this line were not resistant to the disease.

The first observation of the 'appearance' of the agent in susceptible mice that had been free of the agent was seen in the Ax line where only brother-to-sister matings had been continued (19). In this instance the female died noncancerous, but her progeny had cancer, and, by continuing the line for several generations, a high incidence was obtained, and it was determined that the mice had the agent. Several other examples are given above to show that females may suddenly become "infected," even though they have been crossed with males without the agent. Theories may be suggested to explain these observations, but they cannot be supported by experimental evidence.

Studies on the genesis of mammary cancer in mice, and especially the demonstration of mammary tumor agent, have served as the basis for surveys to ascertain whether there might be a nursing influence for breast cancer in humans. Although there are no critical data that such may be the case, since these observations were made on mice, the interpretation given to the work in animals should also be applied to the data for humans. This involves a consideration of the various routes by which such an agent may be transferred.

In addition to being transferred by nursing, it has been found that the agent may be introduced by the injection of extracts of either normal or cancerous tissues from infected mice, including whole blood (46, 47), resuspended blood cells, or serum (23), and it is now definite that the male may transfer the agent to females at the time of coitus. In addition to these possible methods of the transmission of the agent in mice, kissing might have to be included if a comparable agent existed

in humans. Under these circumstances it seems improbable that such an agent might be demonstrated in humans, even if one might be present and involved only in the genesis of one or more types of breast cancer.

SUMMARY

Experimental data have been presented on the transmission by males of cancer stocks of the agent, usually transferred in the milk, for spontaneous mammary cancer in mice.

The infected females may become cancerous or remain noncancerous, but they transfer the agent in their milk to their progeny.

The offspring born to females before they become infected have a low incidence of mammary cancer, but after the mothers become infected their progeny have a high incidence.

The agent may be recovered, as demonstrated by biological assay, from the mammary tumors that develop in either the infected females or their progeny.

Females of strains without the agent differ in their sensitivity to infection by males.

Males of cancerous strains vary in their ability to infect females of either the same or other stocks.

Based upon small numbers, 59 per cent of the females of one inbred strain may develop mammary cancer following infection with the agent by males, while others may become infected but die noncancerous.

Other observations are cited where the agent "appeared" in the progeny born of parents neither of which possessed the agent.

These data are discussed in relation to the interpretation of material obtained to determine whether there may be a nursing influence involved in the genesis of breast cancer in humans.

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Tissue Culture Screening of Purines and Purine Nucleosides for Selective Damage to Mouse Sarcoma Cells*

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The agent 2,6-diaminopurine is much more toxic to mouse sarcoma cells than to mouse embryonic heart or skin cells in tissue culture (10, 11). However, Crocker mouse Sarcoma 180 in mice was not inhibited by 2,6-diaminopurine administered at a level of 60–70 mg/kg (38), and certain normal tissues in the whole animal were rather sensitive to the agent (33). Therefore, a series of purines was tested against Sarcoma 180 cells *in vitro* in order to find agents of greater selectivity of damage to the sarcoma cells and to establish more precisely the nature and position of substituents on the purine nucleus for maximum effectiveness. The most effective purines were adenines substituted in position 2.

Purine nucleosides were also tested because of the hypothesis that purines may be conjugated with pentose as a first step in their incorporation into nucleic acid (7, 36). It was not to be inferred that effects noted with purines or purine nucleosides were necessarily referable to interference with nucleic acid metabolism, in view of the many biochemically active intracellular groupings in which adenine or adenosine, for example, normally occur.

The purine nucleosides tested included naturally occurring nucleosides as well as synthetic variants in which the purine or sugar moiety or both were altered. The most toxic nucleosides were adenosine analogs substituted in position 2 of the purine component. Selectivity of damage to Sarcoma 180 cells was much less pronounced.

MATERIALS AND METHODS

The method employed was a modification of that described by Ormsbee, Cornman, and Berger (32). The culture vessels were roller tubes 16 × 150 mm. In the bottom third of each tube was placed a row of six explants of healthy Crocker mouse Sarcoma 180 about 1 or 2 mm. in diameter. On the opposite wall of the tube was placed a row of six explants of abdominal skin of AKm mice about two-thirds through embryonic life. The explants were held to the glass by a thin clot of cockerel plasma. The fluid nutrient medium, 1 ml. per tube, was made up of 4 parts Gey's balanced salt solution, 2 parts chick embryo extract, 1 part human placental serum, and 3 parts horse serum. The medium also contained 25 units of penicillin G and 25 μg of streptomycin/ml. The tubes were closed with sterile rubber stoppers and put in a drum rotating 10 times per hour to incubate at 37° C.

After incubation overnight, the cultures were examined microscopically for healthy growth. Defective tubes, if any, were discarded, and the remainder were treated with the agent under test. For this purpose, 0.1 ml. of the fluid medium was withdrawn from each tube, and 0.1 ml. of saline or other suitable vehicle containing the agent in 10 times the desired final concentration was added. The agents were used in several final concentrations, ordinarily from 4.0 to 0.125 μM/ml. There were two or three screening tubes for each concentration, and each experiment also included two or more control tubes to which vehicle alone had been added. The tubes were again closed and returned to incubation in the rotating drum.

When the agents were not immediately soluble in saline, an attempt was made to put them into solution with heating or by means of dilute sodium hydroxide or hydrochloric acid. Some agents, especially in higher doses, were added as suspensions. Some suspensions dissolved when diluted in the medium.

Routine microscopic examination of the cultures in order to determine the extent of damage was carried out about 24 hours after addition of the agents to the tubes. This examination, like the earlier, was made at a magnification of 100 or 200 times. In addition to an estimation of the extent of growth, the observations included a grading of damage on a scale of none through slight and moderate to extensive, largely on the basis of rounding up, granularity, and disintegration of cells.

Experiments were repeated with agents that were of particular interest or for which the concentrations first tried showed no gradient of effect.

RESULTS

All possible combinations of toxicities to embryonic mouse skin cells and to Sarcoma 180 cells were found among the purines. Compounds of equal toxicity to both embryonic and sarcomatous cells were most common. A moderate number of compounds caused greater damage to sarcoma cells than to embryonic at one or more concentrations. A few agents caused greater injury to embryonic cells than to sarcomatous at the same concentration.

The data are presented in the tables in highly

* This work was supported in part by grants from the American Cancer Society; the National Cancer Institute, of the National Institutes of Health, Public Health Service; and the Damon Runyon Memorial Fund for Cancer Research.

Received for publication September 12, 1951.

TABLE 1

TOXICITIES OF PURINES IN TISSUE CULTURE

COMPOUND*	THRESHOLD OF MODERATE DAMAGE (μM/ML) Embryo skin	Sarcoma 180
2-Aminopurines:		
2-aminopurine[1]	over 3.3	over 3.3
2-amino-6-thiolpurine[1]	2.0	2.0
2-amino-6,8-dithiolpurine[1]	over 2.0	2.0
2-amino-6-chloro-8-*p*-chlorophenylpurine[1] (18)	2.0	over 2.0
2-amino-6-chloro-8-(2′,4′-dichlorophenyl)purine[1] (18)	over 2.0	over 2.0
2-amino-6-chloro-8-*p*-bromophenylpurine[1] (18)	over 2.0	over 2.0
2-amino-6-chloro-8-*o*-bromophenylpurine[1] (18)	2.0	over 2.0
2-amino-6-bromo-8-*p*-chlorophenylpurine[1] (18)	over 2.0	over 2.0
2-Amino-6-hydroxypurines:		
guanine	over 2.0	over 2.0
8-hydroxyguanine[1]	over 4.0	4.0
8-thiolguanine[1]	over 2.0	over 2.0
8-methylthioguanine[1]	over 2.0	over 2.0
8-methylguanine[1]	over 2.0	over 2.0
8-phenylguanine[1] (18)	over 2.0	over 2.0
8-*p*-nitrophenylguanine[1] (18)	over 2.0	over 2.0
8-*p*-chlorophenylguanine[1] (18)	over 2.0	over 2.0
6-Aminopurines:		
adenine sulfate[2]	over 4.0	4.0
2-hydroxyadenine[5]	over 2.0	over 2.0
2-methoxyadenine[3]	over 2.0	2.0
2-methyladenine[4] (3)	over 4.0	0.5
2-chloroadenine[3]	over 4.0	0.025
2-iodoadenine[3]	4.0	0.5
2-thioladenine[1]	over 2.0	over 2.0
2-methylthioadenine[1]	over 2.0	over 2.0
2-ethylthioadenine[1]	over 2.0	over 2.0
2-*iso*-propylthioadenine[1]	over 1.7	over 1.7
2-*n*-butylthioadenine[1]	over 2.0	over 2.0
2-*sec*-butylthioadenine[1]	0.5	0.5
2-benzylthioadenine[1]	over 2.0	over 2.0
2-methylsulfonyladenine[3]	over 2.0	over 2.0
6-methylaminopurine[1] (19)	2.0	2.0
6-dimethylaminopurine[1] (19)	0.5	0.125
6-ethylaminopurine[1] (19)	over 2.0	0.5
6-acetamidopurine[3] (29)	over 4.0	over 4.0
7-methyladenine[3]	over 2.0	over 2.0
8-thioladenine[1]	over 2.0	over 2.0
8-*p*-chlorophenyladenine[1] (18)	over 4.0	4.0
9-methyladenine[3]	over 2.0	2.0
2-chloro-7-methyladenine[3]	over 2.0	over 2.0
2-chloro-8-hydroxyadenine[3]	over 4.0	over 4.0
2,8-dichloroadenine[3] (23)	over 2.0	over 2.0
2-chloro-9-methyladenine[3]	over 2.0	2.0
2,8-dichloro-9-methyladenine[3]	over 2.0	2.0
2,6-Diaminopurines:		
2,6-diaminopurine lactate[1]	1.0	0.063
2,6-diamino-7-methylpurine[1]	over 6.0	over 6.0
2,6,8-triaminopurine[1]	over 6.0	over 6.0
2,6-diamino-8-hydroxypurine[5]	over 2.0	over 2.0
2,6-diamino-8-thiolpurine[1]	over 2.0	over 2.0
2,6-diamino-8-methylthiopurine[1]	2.0	2.0
2,6-diamino-8-methylpurine[1]	over 2.0	over 2.0
2,6-diamino-8-chloropurine[1]	over 6.0	4.0
2,6-diamino-8-iodopurine[6]	0.5	0.5
2,6-diamino-9-methylpurine[1]	over 4.0	0.5
2,6-diamino-9-β-hydroxyethylpurine[1]	over 2.0	2.0
p-(8-[2,6-diaminopurinyl]methyl)aminobenzoic acid[1]	over 2.0	0.125
2,6-diamino-8-*p*-chlorophenylpurine[1] (18)	over 2.0	2.0
2,6-diamino-8-*o*-chlorophenylpurine[1] (18)	2.0	2.0
2,6-diamino-8-*m*-chlorophenylpurine[1] (18)	over 2.0	2.0
2,6-diamino-8-*m*-bromophenylpurine[1] (18)	2.0	0.125
2,6-diamino-8-*p*-nitrophenylpurine[1] (18)	over 2.0	over 2.0
2,6-diamido-8-(3′,5′-dinitrophenyl)purine[1] (18)	over 2.0	over 2.0
2,6-diamino-8-β-naphthylpurine[1] (18)	2.0	0.5

* Footnotes in tables refer to text footnotes.

TABLE 1—*Continued*

Compound*	Threshold of moderate damage (μM/ml) Embryo skin	Sarcoma 180
2,6-diamino-9-*p*-nitrophenylpurine[1] (18)	over 2.0	2.0
2-dimethylamino-6-aminopurine[3] (1)	over 4.0	2.0
2-amino-6-dimethylaminopurine[1]	over 2.0	over 2.0
2-amino-6-diethanolamino-8-*p*-chlorophenylpurine[1] (18)	2.0	2.0
2,6-diacetamidopurine[3] (15)	over 4.0	over 4.0
2,6-Dihydroxypurines:		
8-methylxanthine[1]	over 2.0	over 2.0
theophylline[2]	over 2.0	over 2.0
7-chlorotheophylline[2]	2.0	2.0
7-(2′,3′-dichloropropyl)theophylline[8]	over 2.0	over 2.0
7-dibenzylaminoethyltheophylline[8]	over 2.0	over 2.0
8,8′-ethylenediaminoditheophylline[3]	over 2.0	2.0
2,6-diketo-3,7-dimethyl-4,5-dihydroxypurine[10]	over 2.0	over 2.0
1-propyltheobromine[7]	over 2.0	2.0
1-(2′methoxyethyl)theobromine[7]	over 2.0	over 2.0
1-allyltheobromine[7]	over 2.0	2.0
1-methallyltheobromine[7]	over 2.0	over 2.0
1-butyltheobromine[7]	over 2.0	2.0
1-(2′-butenyl)theobromine[7]	2.0	2.0
1-*iso*-amyltheobromine[7]	2.0	2.0
caffeine	over 2.0	2.0
8-chlorocaffeine[9]	2.0	2.0
8-ethoxycaffeine[11]	4.0	4.0
8-*iso*-amyloxycaffeine[11]	over 4.0	over 4.0
6-Hydroxypurines and miscellaneous:		
6-thiolpurine[1] (19)	over 4.0	over 4.0
6-methylthiopurine[1] (19)	over 2.0	0.5
6-chloropurine[13]	over 4.0	over 4.0
2-chlorohypoxanthine[3]	over 2.0	over 2.0
2-methylthiohypoxanthine[1]	over 2.0	over 2.0
2,6-diiodopurine[3] (24)	0.5	0.125
2-chloro-7-methylhypoxanthine[1]	over 2.0	2.0
2,6-dichloro-7-methylpurine[1]	0.1	0.1
2,6-dichloro-8-hydroxypurine[1]	over 2.0	over 2.0
2,8-dichlorohypoxanthine[3]	over 2.0	over 2.0
2,6,8-trichloropurine[1]	2.0	2.0

condensed form. Concentrations of agents are given at which moderate damage to the cultured cells began. If the concentrations tested were all too low to cause intermediate damage, this is indicated. For the sake of compactness, the tables do not indicate whether the original material came as a salt, hydrate, or free base.

2-Aminopurines.—The 2-aminopurines were not highly toxic (Table 1). At 2.0 μM/ml, 2-amino-6-thiolpurine[1] damaged Sarcoma 180 cells extensively and embryonic cells moderately, while 2-amino-6, 8-dithiolpurine[1] was somewhat less injurious. Both 2-amino-6-chloro-8-*p*-chlorophenylpurine[1] (18) and 2-amino-6-chloro-8-*o*-bromophenylpurine[1] (18) were more harmful to embryonic skin cells than to sarcoma cells. The other four compounds showed little activity against either tissue.

2-Amino-6-hydroxypurines.—Guanine and its derivatives were nearly all innocuous. Only two guanines of eight tested caused any damage. 8-Hydroxyguanine[1] brought about moderate injury to Sarcoma 180 cells and slight injury to embryonic skin cells at 4.0 μM/ml. 8-Phenylguanine[1] damaged Sarcoma 180 cells slightly at 2.0 μM/ml.

6-Aminopurines.—Derivatives of adenine were available in larger numbers and included some compounds of highly selective toxicity.

Adenine sulfate[2] was itself slightly toxic to both tissues at intermediate concentrations and moderately toxic to Sarcoma 180 cells at 4.0 μM/ml.

Certain adenines substituted in position 2 alone were particularly effective. 2,6-Diaminopurine is, of course, to be included in this group. The agent 2-chloroadenine[3] showed a wider spread in toxicity to the two tissues than did any other purine. Damage to cells growing out from explants of embryonic skin was, at most, only slight over the whole range from 0.0016 to 4.0 μM/ml. Sarcoma 180 cells suffered extensive damage at concentrations as low as 0.05 or 0.025 μM/ml. The correspondingly effective concentration for 2,6-diaminopurine lactate[1] was 0.1 μM/ml, or 2–4 times as great. With this spread in active concentrations, 2-chloroadenine was the most effective agent in the entire series of purines tested. Figures 1–4 illustrate its effect.

Other 2-substituted adenines differentially toxic to Sarcoma 180 cells included 2-methyladenine (3),[4] 2-iodoadenine,[3] and 2-methoxyadenine,[3] in order of diminishing selectivity. The

[1] These compounds were kindly supplied by Miss Gertrude B. Elion and Dr. George H. Hitchings of the Wellcome Research Laboratories.

[2] These compounds were kindly supplied by Merck & Co.

[3] These compounds were kindly supplied by Dr. George B. Brown and Dr. John Davoll of the Bio-organic Chemistry Section of the Sloan-Kettering Institute.

[4] The agent 2-methyladenine was prepared by Dr. G. B. Brown and Dr. John Davoll of the Sloan-Kettering Institute from an intermediate kindly provided them by Dr. Howard Bond of the National Institutes of Health.

lack of toxicity of 2-hydroxyadenine,[5] or isoguanine, was noteworthy.

Adenines substituted with sulfur-containing groups at position 2 were for the most part of low and nondifferential toxicity.

Substitution on the 6-amino group in adenine produced some agents selectively damaging to sarcoma cells. 6-Dimethylaminopurine[1] (19) and 6-ethylaminopurine[1] (19) were selectively toxic, while 6-methylaminopurine[1] (19) and 6-acetamidopurine[3] (29) were not.

The remaining adenines, most of which were polysubstituted, were of approximately the toxicity of adenine itself.

Reduction in toxicity was not so great with aromatic substituents on C_8 of 2,6-diaminopurine, and the majority of such derivatives tested caused selective damage to Sarcoma 180 cells at some concentration. These included *p*-(8-[2,6-diaminopurinyl]methyl) aminobenzoic acid,[1] an analog of pteroic acid, as well as some less toxic 8-halophenyl derivatives[1] (18) of 2,6-diaminopurine. Substituents on the amino groups caused changes in activity, as seen in the reduced toxicity of 2-dimethylamino-6-aminopurine[3] (1), 2-amino-6-dimethylaminopurine,[1] and 2,6-diacetamidopurine[3] (15).

2,6-Dihydroxypurines.—Xanthine, as well as hypoxanthine had earlier been observed to be innocuous in mouse tissue cultures (11). The eighteen methylated xanthines examined ran the gamut from selectivity of damage to Sarcoma 180 cells to selective damage to embryonic mouse skin cells in culture. None was highly toxic.

TABLE 2

TOXICITIES OF PURINE NUCLEOSIDES IN TISSUE CULTURE

COMPOUND*	THRESHOLD OF MODERATE DAMAGE (μM/ML)	
	Embryo skin	Sarcoma 180
adenosine, or 9-β-D-ribofuranosyladenine	over 6.0	over 6.0
guanosine, or 9-β-D-ribofuranosylguanine	over 2.0	over 2.0
inosine, or 9-β-D-ribofuranosylhypoxanthine	over 4.0	over 4.0
xanthosine, or 9-β-D-ribofuranosylxanthine[12]	over 4.0	over 4.0
crotonoside, or 2-hydroxy-9-β-D-ribofuranosyladenine[13]	0.5	0.5
2,6-diamino-9-β-D-ribofuranosylpurine[12] (15)	2.0	2.0
2-acetylamino-9-β-D-ribofuranosyladenine[12] (15)	over 2.0	over 2.0
2-methyl-9-β-D-ribofuranosyladenine[12]	over 4.0	over 4.0
2-methylthio-9-β-D-ribofuranosyladenine[12]	over 2.0	over 2.0
2-chloro-9-β-D-ribofuranosyladenine[12] (17)	0.125	0.125
2,8-dichloro-9-β-D-ribofuranosyladenine[12] (16)	over 2.0	over 2.0
2′,3′-*iso*-propylidene adenosine[12] (30)	over 2.0	over 2.0
2′,3′-*iso*-propylidene guanosine[12] (31)	over 4.0	over 4.0
9-β-D-ribopyranosyladenine[12] (2)	over 2.0	over 2.0
2,6-diamino-9-β-D-ribopyranosylpurine[12]	over 2.0	over 2.0
2-hydroxy-9-β-D-ribopyranosyladenine[12] (impure)	over 4.0	over 4.0
9-β-D-glucopyranosyladenine[12] (15)	over 2.0	over 2.0
9-β-D-glucopyranosylguanine[12] (15)	over 2.0	over 2.0
2-hydroxy-9-β-D-glucopyranosyladenine[12] (14)	over 2.0	over 2.0
2,6-diamino-9-β-D-glucopyranosylpurine[12] (15)	over 2.0	over 2.0
2-chloro-9-β-D-glucopyranosyladenine[12] (25)	over 2.0	over 2.0
2,8-dichloro-9-β-D-glucopyranosyladenine[12] (16)	over 2.0	over 2.0
9-α-D-arabofuranosyladenine[12] (12)	over 2.0	over 2.0
9-α-L-arabofuranosyladenine[12] (12)	over 2.0	over 2.0
2,8-dichloro-9-α-D-arabofuranosyladenine[12] (12)	over 2.0	over 2.0
2,8-dichloro-9-α-L-arabofuranosyladenine[12] (12)	over 2.0	over 2.0
9-β-D-xylofuranosyladenine[12] (impure)	0.5	0.5
2,6-diamino-9-β-D-xylofuranosylpurine[12]	0.5	2.0

* Footnotes in tables refer to text footnotes.

2,6-Diaminopurines.—No substituted 2,6-diaminopurine tested was as toxic to either tissue *in vitro* as the parent compound. Presence of a nonaromatic substituent on ring atoms C_8, N_7, or N_9 of 2,6-diaminopurine caused great loss of toxicity, with the exception of the 8-iodo[6] and 9-methyl[1] compounds.

Miscellaneous purines.—Of the hypoxanthines and miscellaneous purines remaining in Table 1, two are outstanding for high toxicity: 2,6-dichloro-7-methylpurine[1] and 2,6-diiodopurine[3] (24). The latter compound was significantly more toxic to Sarcoma 180 cells, as was 6-methylthiopurine[1] (19).

Purine nucleosides.—Like adenosine, guanosine, inosine, and xanthosine,[12] most of the nucleosides did not appear toxic in this test (Table 2). Only five nucleosides of 28 caused morphological damage greater than slight. Their structural formulas and that of adenosine are given in Chart 1. In order of decreasing toxicity, these five were 2-chloro-9-β-D-ribofuranosyladenine (2-chloroadenosine)[12] (17), 2-hydroxy-9-β-D-ribofu-

[5] These compounds were kindly supplied by Dr. G. B. Brown and Dr. L. F. Cavalieri of the Bio-organic Chemistry Section of the Sloan-Kettering Institute.

[6] This compound was kindly provided by Dr. Howard Skipper of the Southern Research Institute.

[7] These compounds were kindly supplied by Monsanto Chemical Co.

[8] These compounds were kindly supplied by Endo Products, Inc.

[9] This compound was kindly supplied by Parke, Davis & Co.

[10] This compound was kindly provided by Abbott Laboratories.

[11] These compounds were kindly provided by G. D. Searle & Co.

[12] These purine nucleosides were kindly provided by Dr. G. B. Brown, Dr. John Davoll, and Mr. Bertram Lowy of the Bio-organic Chemistry Section of the Sloan-Kettering Institute.

ranosyladenine (crotonoside),[13] an impure preparation of 9-β-D-xylofuranosyladenine,[12] 2,6-diamino-9-β-D-xylofuranosylpurine,[12] and 2,6-diamino-9-β-D-ribofuranosylpurine (2-aminoadenosine)[13] (15).

2-Chloroadenosine had a threshold of extensive damage to both embryonic and sarcomatous mouse cells at about 0.5 μM/ml. Toxicity was lost with an additional 8-chloro group on the molecule, as in 2,8-dichloro-9-β-D-ribofuranosyladenine[12] (16).

Crotonoside was extensively damaging to both tissues at 2.0 μM/ml, but at lower concentrations it damaged embryonic mouse cells somewhat more than sarcoma cells (11).

2-Aminoadenosine was of interest because of its relation to 2,6-diaminopurine. It was considerably less toxic than the free purine and much reduced in selectivity of damage to Sarcoma 180 cells. The effects of adenosine and guanosine on the toxicity of 2-aminoadenosine are discussed elsewhere (8, 9). Acetylation of the 2-amino group in this compound to give 2-acetylamino-9-β-D-ribofuranosyladenine[12] (15) destroyed the activity of the compound.

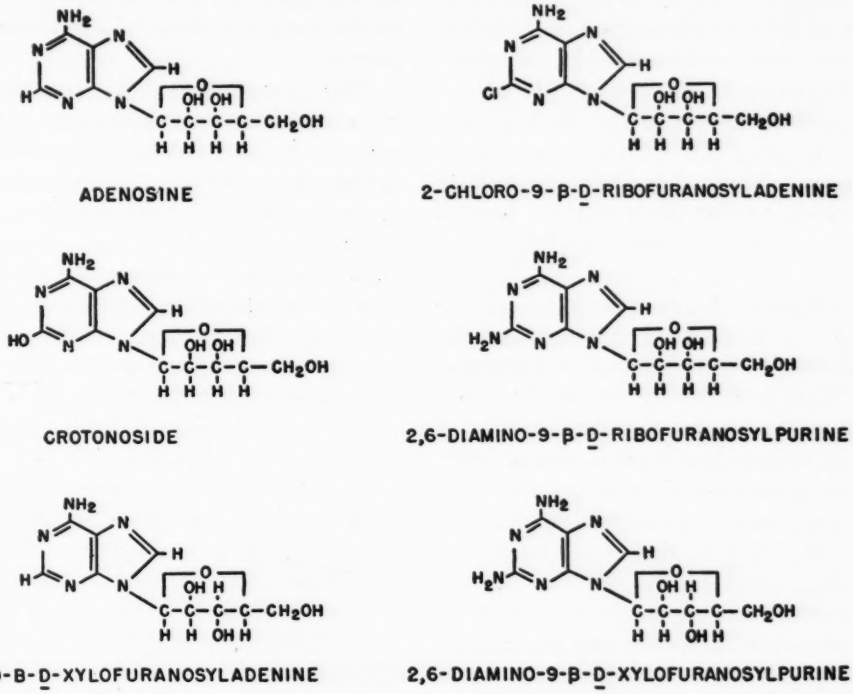

CHART 1.—Structural formulas of adenosine and five toxic purine nucleosides

The two nucleosides containing xylose caused extensive damage to cells of both tissues at 2.0 μM/ml. At lower concentrations their effects were somewhat irregular.

A diminution in toxicity on changing the sugar configuration was seen in 2,6-diamino-9-β-D-ribopyranosylpurine[12] as compared to 2-aminoadenosine and in 2-hydroxy-9-β-D-ribopyranosyladenine[12] as compared with crotonoside.

The glucopyranosylpurines and arabofuranosylpurines displayed no material toxicity.

DISCUSSION

Compounds causing greater damage in tissue culture to Sarcoma 180 cells than to embryonic mouse skin cells were to be found in every large group of purines tested. There were few such purines of moderate or greater activity among the 2-aminopurines, the 2-amino-6-hydroxypurines (guanines), the 6-hydroxypurines (hypoxanthines), or the 2,6-dihydroxypurines (xanthines). It was among the 6-aminopurines (adenines) and the 2,6-diaminopurines that the selectively damaging compounds were most prominent. The peak of differential activity was reached in certain adenines substituted at position 2 only, and particularly in those compounds whose 2-substituent was small and simple. Additional substituents at other positions reduced the effectiveness of 2-substituted adenines.

The 2,6-diaminopurines may formally be reckoned among the 2-substituted adenines. With them, too, there was loss of activity as substituents were added. It is noteworthy that 8-substituted 2,6-diaminopurines were not invariably ineffective, as had been reported for such compounds when tested against transmitted mouse leukemia (26, 37). Activity against Sarcoma 180 cells was often maintained when the compound was a member of the new class (22) of 8-arylpurine inhibitors.

Just as 2,6-diaminopurine was found to be an antagonist of adenine in tissue cultures (11), so 2-chloroadenine has been shown to be an anti-adenine substance (8). This suggests that in mouse cell metabolism there may be no special significance to the 2,6-diamino configuration *per se*, par-

[13] These compounds were kindly supplied by Dr. G. B. Brown and Dr. Aaron Bendich of the Bio-organic Chemistry Section of the Sloan-Kettering Institute.

ticularly in view of the findings that the analogous 2,4-diamino configuration in pteridines (38) and the 5,7-diamino grouping in triazolo-(d)-pyrimidines[14] are not very toxic in tissue cultures.

To have the 2,6-diaminopurines placed among the other 2-substituted adenines does not eliminate the possibility that there might be mechanisms of action peculiar to 2,6-diaminopurines. We refer here to the theory of 2,6-diaminopurine toxicity (7), based on the observation that this purine is a precursor of nucleic acid guanine in the rat (5, 7). We also have reference to the apparently singular position of 2,6-diaminopurine in its effect on the mechanisms concerned with the interconversion of the physiological purines (4, 21, 22).

Attention should be drawn to the finding that the selectively damaging 8,8′-ethylenediaminoditheophylline was not blocked by adenine sulfate (8). Very likely there are for these purines several pathways of action in the destruction of Sarcoma 180 cells.

The most effective pathway seems to be one concerned with the metabolism of adenine, and the most effective purine yet tested is 2-chloroadenine. It is of interest that new highly effective agents were found, not only among the derivatives of 2,6-diaminopurine where they were earlier sought, but also among other 2-substituted adenines.

Many of the purines whose toxicities in tissue culture are here reported have been studied for their effects on the growth of *Lactobacillus casei* in media lacking in or containing known amounts of physiological purines and folic acid (20, 21, 22, 27). While the similarities in response of the mouse cells and *L. casei* cultures were striking, some points of difference were also found. These may have been based in part on metabolic differences between mouse cells and *L. casei* cells, and in part on the natural origin of the mouse tissue culture medium and the possibility that it contained substances antagonistic to the tested purine inhibitors.

While the nucleosides were in general less toxic to mouse cells than the corresponding free purines, the relative toxicities of some of the nucleosides bore a rough correspondence to those of the free purines.

The more highly toxic purine nucleosides included agents whose purine moieties when free were toxic adenines with substituents, if any, only in position 2. 2-Chloroadenine and 2,6-diaminopurine were highly toxic. Although isoguanine, or 2-hydroxyadenine, caused "adenine kidney" in rats (6), no damage from it was observed in tissue culture, perhaps because of low solubility. The toxicity of adenine has been recognized (6, 35) and could be seen at higher concentrations in tissue culture.

The possibility that the toxicity of purine nucleosides resided only in the toxicity of purines released from them appears to be negated by the greater toxicity of certain nucleosides than of their free purines. Considerations of solubility and permeability would, of course, enter into a complete discussion of this question. Effectiveness of the whole nucleoside molecule was also indicated by the fact that some toxic nucleosides were blocked by adenosine as well as by adenine (8), in contrast to the ineffectiveness of adenosine against the free purine (11).

The more highly toxic purine nucleosides contained a ribose or xylose sugar of furanose configuration. A low toxicity characterized the ribopyranosylpurines, glucopyranosylpurines, and arabofuranosylpurines tested.

These facts suggest that a fairly close structural correspondence to adenosine was necessary for activity of nucleosides in this test. The inference that the toxic nucleosides might be antagonists of adenosine was borne out in the case of 2-aminoadenosine with both mouse embryo skin and Sarcoma 180, in the case of 9-β-D-xylofuranosyladenine with possibly both tissues, and in the case of 2-chloroadenosine with embryo skin but perhaps not with Sarcoma 180 (8). Crotonoside, on the other hand, was not blocked by adenosine (8) or by guanosine (11) in mouse tissue cultures, or by adenosine in rat tissue cultures (11).

In addition to an antimetabolite action, another explanation of nucleoside toxicity might be proposed. Because free 2,6-diaminopurine is known to be a precursor of polynucleotide guanine in the rat (5), while free guanine is not detectably so (13, 34), it has been pointed out that conjugation of 2,6-diaminopurine with ribose or desoxyribose might be a first step in its change to nucleic acid guanine (7). Hence, the hypothesis of 2,6-diaminopurine toxicity proposed by Bendich, Furst, and Brown (7) could possibly be extended to 2-aminoadenosine, at least, among the adenosine analogs. The hypothesis conceives of a disturbance in the structural integrity and therefore in the functional ability of nucleic acids in tissues flooded by a specific precursor of one purine.

If the toxicity of 2-aminoadenosine were explicable on this basis, at least in part, then it might represent a small portion of the toxicity of free 2,6-diaminopurine. The observation of greater toxicity of the free purine than of the riboside suggests that either (*a*) purine is not necessarily incorporated into nucleic acid via its nucleoside or (*b*) there are additional mechanisms of action of

[14] Unpublished results.

2,6-diaminopurine besides disturbance of nucleic acid metabolism in this manner.

An important aspect of the toxicity of the purine nucleosides was the low differential in their damage to the two tissues. The extent of damage sustained by fetal mouse skin cells in culture approximated that suffered by Sarcoma 180 cells. This is in contrast to the result with free purines. *In vitro*, the purine nucleosides were less selective and generally less active chemotherapeutic agents than were the purines.

It is to be noted that the observations recorded in the tables related to gross toxic effects seen after but 1 day of exposure to the agents. Damage resulting from longer exposure is not indicated. 2-Methyladenine at 4.0 μM/ml produced a differential effect after 2 days, with damage to Sarcoma 180 cells being moderate to extensive and that to embryonic skin cells being one grade lower. Cultures fixed and stained after 2 days exposure to adenosine, inosine, and guanosine at higher concentrations revealed partial inhibition of growth and of mitosis, with some pyknotic cells (9).

SUMMARY

1. Comparative toxic effects of 96 purines and 28 purine nucleosides on embryonic mouse skin cells and Crocker mouse Sarcoma 180 cells in tissue culture are reported.

2. Compounds causing the greatest differential damage to Sarcoma 180 cells were most prominent among the adenines and the 2,6-diaminopurines. The 2-aminopurines, guanines, hypoxanthines, and xanthines were less effective.

3. The most effective compounds were certain 2-substituted adenines, particularly 2-chloroadenine, 2,6-diaminopurine, 2-methyladenine, 2-iodoadenine, and certain 2,6-diamino-8-arylpurines.

4. Additional substituents at other positions reduced the effectiveness of 2-substituted adenines.

5. The five nucleosides that caused more than slight damage in 1 day of exposure were ribofuranosyladenines or xylofuranosyladenines substituted, if at all, only in position 2 of adenine. The most toxic was 2-chloroadenosine.

6. Purine nucleosides, with the major exception of 2-hydroxyadenosine, tended to be less toxic to Sarcoma 180 cells than the corresponding free purines. For example, 2-aminoadenosine was about $\frac{1}{10}$th as toxic to Sarcoma 180 cells as was 2,6-diaminopurine. Some nucleosides were more toxic to embryonic cells than were the free purines.

7. Consequently, embryonic and sarcomatous mouse cells were damaged to about the same extent by purine nucleosides.

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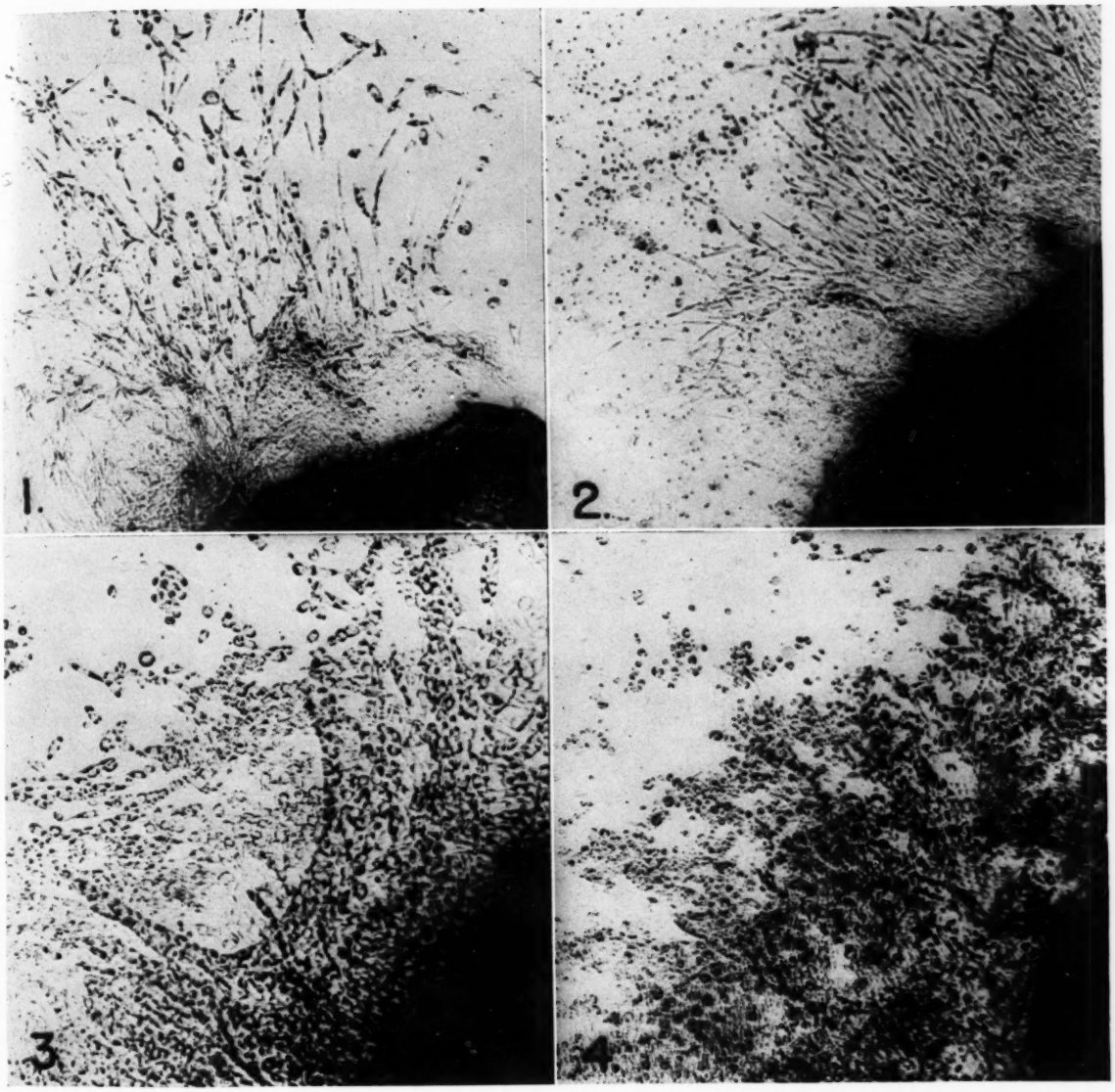
Fig. 1.—Control roller tube culture of embryonic AKm mouse skin. Mag. ×75.

Fig. 2.—Roller tube culture of embryonic AKm mouse skin after 24 hours treatment with 2.0 μM/ml of 2-chloroadenine. The particulate debris in upper left is undissolved drug. The cells appear unharmed. Mag. ×75.

Fig. 3.—Control roller tube culture of Crocker mouse Sarcoma 180. Mag. ×75.

Fig. 4.—Roller tube culture of Sarcoma 180 after 24 hours treatment with 0.025 μM/ml of 2-chloroadenine. The cells are badly damaged, and many are disintegrating. Mag. ×75.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Mitochondrial Population in Mammalian Cells

I. Description of a Counting Technic and Preliminary Results on Rat Liver in Different Physiological and Pathological Conditions*

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INTRODUCTION

The evaluation of the total number of mitochondria in cells has been the subject of a few investigations. Attempts have been made in the past to count them in cells (3, 18). These methods were histological and are considered at the present time as tentative. Since centrifugation has been applied to isolate particulates from the cell, the yield in grams of mitochondrial material has been intensively investigated (10, 12–15). Schneider and Hogeboom have discussed this matter recently (17). However, no accurate methods have been devised to count mitochondria. Harman presented briefly a method of counting the particulate "cyclophorase" (6). This has been, to our knowledge, the only attempt to express enzymatic activities in relation to the number of particles. We have dismissed Harman's method *a priori* for two reasons. First, it is too elaborate to be practical; and, second, its accuracy is not indicated.

Variation in the population of mitochondria in the liver cell has been qualitatively estimated (13). Furthermore, Potter, Price, Miller, and Miller (10) have proposed a hypothetical mechanism of carcinogenesis in terms of the deletion of cytoplasmic particles or of an enzyme contained therein. Schneider and Hogeboom have stressed the necessity of determining the yield in terms of the number of mitochondria (17).

We present a method for the estimation of the number of mitochondria or large granules in homogenate, or when isolated in 0.25 M or in 0.88 M sucrose from a known number of normal rat liver cells. It has been shown that mitochondria, isolated as such, seem to retain the morphological and staining properties characteristic of normal intracellular liver mitochondria (8).

The method consists essentially in counting fresh mitochondria dispersed at random in a known volume of suspension, and it can be applied to the study of the number of mitochondria per normal rat liver cell. Preliminary results are herewith reported for the liver cell in other physiological and pathological conditions.

METHODS

Isolation of mitochondrial particulate.—The animals (Wistar rats) were killed by a blow on the head and were immediately decapitated. Exsanguination was completed by holding the animal by the tail and massaging the chest and back. The whole liver was excised, placed on cracked ice, and chilled for 2 minutes, then rolled on filter paper to sponge off water and blood. The tissue was immediately minced in a cold (5° C.) plexiglas squeezer. Collected liver pulp, thus prepared, was mixed by hand. An amount of 2.5 gm. was rapidly weighed in a 20-ml. beaker. Five ml. of ice-cold 0.25 M salt-free sucrose (or other medium) was immediately added to the weighed amount of liver pulp and again mixed. The suspension was poured into an ice-cold 15-ml. Potter homogenizer tube. Five more ml. of medium was used to rinse the beaker. The time interval of homogenization with a pestle turning at a speed of 300 r.p.m., was 2½ minutes, which gave a maximum cell rupture with the homogenizer used in this work. This was controlled by determinations of enzymatic activity (1) and by phase and light microscopic examination. The suspension was then poured into a 25-ml. volumetric flask and diluted to the mark with the medium.

Exactly 20 ml. of the resulting 10 per cent homogenate, prepared in 0.25 M or 0.88 M salt-free sucrose, was immediately centrifuged to isolate a nuclear (N),[1] a mitochondrial (M) and a supernatant (S) fraction. The centrifugal fractionation was carried out as described by Schneider and Hogeboom (16), except that the (S) fraction obtained after isolation of (N) and (M) was not further fractionated. After the fractions were isolated, they were immediately diluted with the medium to the following concentrations, as compared to the original weight of tissue: (N), 20 per cent; (M), 20 per cent; (S), 4 per cent, i.e., 1 ml. of the (N) or (M) fraction contained 20 per cent of the particles

[1] The nuclear fraction is contaminated with blood cells, cell debris, and mitochondria.

* This work was supported by a grant from the National Cancer Institute of Canada.

Received for publication November 27, 1951.

from 1 gm. of tissue or 0.2 gram-equivalent of nuclei or mitochondria. Servall centrifuges SS-1 and SS-2 were employed throughout. The temperature of the tissue or the particulate suspension never exceeded 15° C.

Purity of the (M) fraction was established by total nitrogen and pentosenucleic acid determinations, by histological examination of smears, and by phase and electron microscopy.

The mean total nitrogen content of eight normal rat liver mitochondrial fractions isolated in 0.25 M sucrose was found to be 6.4 ± 1.4 mg. per gram of fresh liver and corresponded to 21.4 ± 1.8 per cent of the total nitrogen content of liver. The mean pentosenucleic acid phosphorus content, estimated on the mitochondrial fraction from five normal livers, was 123 μg. and corresponded to 15.6 per cent of the total liver PNA phosphorus. This confirmed earlier results obtained on pure (M) fractions (17). Electron microscopic studies confirmed the fact that only one type of particulate was present. Fig. 1 illustrates the homogeneity of the suspension. Small debris seen in the photograph are probably parts of broken mitochondria, since a few disintegrating mitochondria are always present, as shown in Figs. 2 and 3. Examination of the (S) fraction reveals this fraction to be free of mitochondria.

Counting technic.—The apparatus adopted for counting mitochondria was the Petroff-Hausser bacteria counter. The chamber consisted essentially of 400 prisms,[2] each of 0.05 × 0.05 × 0.02 mm., thus having a volume of 5×10^{-8} cm³. A Zeiss-Winkle binocular microscope with phase contrast (Ph2) attachment was used. Oil immersion 100× and 1.3 numerical aperture was employed in conjunction with 12.5 Hygens eyepieces. Köhler illumination, with a Wratten filter No. 22, was utilized. Four glass fibers, cemented at right angle with glassware cement, were fixed in one eyepiece. This reticule was designed to be optically superimposed on a square of the chamber.

Mitochondria suspended in 0.25 M or in 0.88 M sucrose could not be counted in the chamber because of Brownian movement of the particles. All attempts to stop this movement or to sediment mitochondria chemically proved unsuccessful. It was found possible, however, to diminish this movement greatly by diluting the mitochondria with 1.8 M sucrose. When kept in this medium at 0–5° C., they did not sediment, and they maintained their original form. Their number seemed to remain constant for a period of as long as 3 days. Fading was the only visible change in the particles after such a storage period.

The technic adopted for counting was as follows: A suspension was prepared by diluting 0.5 ml. of a 20 per cent mitochondrial suspension to 50 ml. with 1.8 M sucrose, giving a final concentration of 0.2 per cent, as compared to the original tissue homogenate. After thorough mixing, an aliquot was pipetted and held between the hands until it attained room temperature. Movement of the particles due to the expansion of the liquid in the chamber was thus avoided. The chamber was finally filled by capillary attraction. Five minutes after the introduction of the specimen in the chamber, movement had usually ceased; the particles, dispersed at random, were ready to be counted.

Since the particles did not sediment in the chamber, they were counted from the base to the top of a prism. This was conveniently done by focusing the objective on the base of a prism, followed by a very slow ascending movement of the objective to the upper surface of the same prism. The reticule in the eyepiece served as the true limit of the surface of counting. With such a slow movement of the objective, mitochondria appeared and disappeared progressively. They were counted when perfectly focused. Since the volume of a prism and the concentration of the mitochondrial suspension were known, the total number of particles in the (M) fraction per gram of fresh liver could be computed. Dealing with 0.2 per cent concentrations, the value for the total number of particles present in the (M) fraction per gram of fresh tissue was obtained by multiplying the mean number of mitochondria counted in one prism by the factor 10^{10}.

[2] By "prism" is meant the volume between the cover glass of the chamber and the surface of one square engraved on the plate.

RESULTS

The number of granules found in the (M) fraction from nine normal rat livers, isolated in 0.25 M or in 0.88 M sucrose, lies within the limits of 29.0 to 30.2 × 10^{10}, with a probability of 0.975. The mitochondria contaminating the nuclear fraction were counted from eight normal rat liver (N) fractions. With the same probability, the number lies within the limits of 2.84–4.18 × 10^{10}, which gives, per gram of fresh rat liver tissue, a total ranging from 31.84 to 34.18 × 10^{10}. Counts in the nuclear fraction are made in the same manner as with the mitochondrial fraction, but at a concentration of 1 per cent. Nuclei at this concentration are so scarce (3 or 4 per 400 prisms) that they do not interfere with mitochondrial counts. These results are presented in detail in Table 1. Results of a standard method of verification when working with particulates are given in Table 2. Summation of counts made on three different (M) and (N) and (S) fractions, isolated from a normal rat, and of those from two regenerating livers agrees with counts made on each of the original liver homogenates. Meticulous care is required to count mitochondria directly in the homogenate, and the method in this case is not so convenient for routine assay.

Preliminary results of the study of the variation of mitochondria population in different pathological and physiological conditions are presented. Table 3 shows results obtained with total liver with hepatoma and with pure hepatoma induced by feeding a semi-synthetic diet (9, diet 3) containing 0.06 per cent of 4-dimethylaminoazobenzene. The number of mitochondria per gram of fresh tissue seems higher in tumor than in normal rat liver. The difference is statistically very significant. This seems confusing, since it is known that the yield of mitochondria expressed as dry weight, protein or/and PNA content per gram of fresh tissue is lower in tumor than in normal tissue (10, 12–15, 17). These data and ours would imply that mitochondria may be much smaller in tumor tissue. In fact, it is apparent, when tumor (M) fraction is examined under the phase microscope, that a greater number of smaller mitochondria is present. Another explanation would be that they are broken down during homogenization, because of an increased fragility. This problem is now under investigation.

It is of interest to know whether the number of mitochondria fluctuates during normal prolifera-

tion, as it appears to do during cancerous growth. Preliminary study of this phenomenon has been undertaken during rat liver regeneration. Partial hepatectomy is performed as described by Higgins and Anderson (7). Results are given in Table 4 for a period of 2 days of regeneration. The total number of mitochondria per gram of fresh tissue lies within the limits of 22.8–28.6 $\times$ 10^{10}. The difference with that of normal rat liver (31.8–34.3 $\times$ 10^{10}) is statistically very significant.

Preliminary results on the influence of diet on the variation of the number of mitochondria in rat liver tissue are given in Table 5. After 6 days of fasting (rat No. 221), the number of mitochondria has increased to a value much higher than for normal rat liver. On a semi-synthetic diet (9, diet 3), the number decreases; the same phenomenon occurs with a diet of cooked polished rice.

Since all results given above are expressed per gram of fresh tissue, this may lead to an erroneous interpretation. The cell as a base of calculation seems much more appropriate. In Table 6 the total number of mitochondria per liver cell, in different conditions, is given. The values for the number of cells per gram of fresh tissue were taken in part from the literature. In normal adult rat liver a mean value of 133 $\times$ 10^6 is obtained in this laboratory. This confirms the results of Price and Laird (11). Since 1 gram of fresh normal rat liver contains about 33 $\times$ 10^{10} mitochondria, a rat liver cell would thus contain approximately 2,500 mitochondria.

In total rat liver with tumor induced by DAB feeding the number of cells seems to decrease. From seven assays, a mean value of 92 $\times$ 10^6 is obtained in this laboratory. Thus, the mean number of mitochondria per cell would be approximately 4,500.

The number of cells per gram of fresh pure liver tumor calculated from DNA concentrations taken from Price *et al.* (11, 12) is approximately 554 $\times$ 10^6. The number of mitochondria per neoplasic cell would fall to approximately 700. From the value of Price and Laird for the number of cells after 2 days of liver regeneration, the number of mitochondria per cell is 2089. After 4 days it appears that the mitochondrial population is half the normal value.

TABLE 1

NO. OF MITOCHONDRIA PER GRAM OF FRESH RAT LIVER IN THE NUCLEAR (N) AND MITOCHONDRIAL (M) FRACTIONS

			Isolated in 0.25 M Sucrose			
		Weight	N fraction		M fraction	
Rat no.	Sex	(gm.)	$\bar{X}\times10^{-10}$	A^*	$\bar{X}\times10^{-10}$	A^*
226	M	180	4.70	±0.30	30.2	±1.80
215	M	170	3.68	±0.24	29.5	±2.14
216	F	167	3.72	±0.12	29.2	±1.35
218	M	167			29.5	±1.45
223	M	150	3.58	±0.14	30.0	±2.03
224	M	200	2.80	±0.62	30.9	±1.70
300	M	180	2.00	±0.21	28.2	±1.70
			Isolated in 0.88 M Sucrose			
1-88M	M	195	4.02	±0.35	29.6	±1.58
3-88M	M	170	3.60	±0.21	29.2	±2.30

* $A = t_{0.025}\frac{S}{\sqrt{n-1}}$. Where $t_{0.025}$ is the Student "t" and $S = \sqrt{\frac{1}{n}\Sigma(X_i - \bar{X})^2}$. X_i represents the individual counts, and $\bar{X}$ the mean value of 26 counts on the same sample.

TABLE 2

NO. $\times$ 10^{-10} OF MITOCHONDRIA PER GRAM OF FRESH RAT LIVER IN THE HOMOGENATE AND IN THE FRACTIONS ISOLATED IN 0.25 M SUCROSE

		Regenerating	
Description	Normal	12 days	12 days
Rat no.	226	25-R	26-R
Sex	male	male	male
Weight (gm.)	180	220	210
Homogenate ($\bar{X}$)	35.50	30.70	26.00
(A)	± 2.06	± 1.09	± 1.52
N Fraction ($\bar{X}$)	4.70	3.92	4.18
(A)	± 0.30	± 0.28	± 0.26
M Fraction ($\bar{X}$)	30.20	25.10	21.50
(A)	± 1.83	± 1.22	± 1.00
S Fraction	none	none	none
Total	34.93	29.02	25.68
Recovery (per cent)	98.3	94.5	98.8

DISCUSSION

Method.—The technic described above was subjected to an elaborate verification. The results were compiled independently by three individuals, and their counts agree with a probability of 0.95. Table 7 includes values obtained with replicate counts done by three different operators (a, b, c) on the same specimen. Each operator did three counts on the same prism. This was repeated with three different prisms (Nos. 1, 2, 3). The difference between the values obtained by each operator is not

TABLE 3

NO. OF MITOCHONDRIA PER GRAM OF FRESH TUMOR TISSUE IN THE NUCLEAR (N) AND MITOCHONDRIAL (M) FRACTIONS ISOLATED IN 0.25 M SUCROSE

Rat no.	Sex	Weight (gm.)	Description	N fraction $\bar{X}\times10^{-10}$	N fraction A	M fraction $\bar{X}\times10^{-10}$	M fraction A
219	F	150	Hepatoma-parenchyma*			36.8	±1.50
220	F	120	Pure hepatoma			35.6	±2.17
222	M	110	Pure hepatoma			33.1	±1.27
	Pool M	120					
	F	160					
225	F	158	Cholangioma-hepatoma	5.10	±0.36	38.2	±4.60
228	Pool M	160	Hepatoma-parenchyma*	5.03	±0.34	35.5	±1.90
	M	150					

* Total liver with tumor.

TABLE 4

NO. OF MITOCHONDRIA PER GRAM OF FRESH REGENERATING RAT LIVER IN THE NUCLEAR (N) AND MITOCHONDRIAL (M) FRACTIONS ISOLATED IN 0.25 M SUCROSE

Rat no.	Sex	Weight (gm.)	Days	N fraction $\bar{X}\times10^{-10}$	N fraction A	M fraction $\bar{X}\times10^{-10}$	M fraction A
221-A	Pool F	130	2			22.0	±1.75
	M	145					
1-R	Pool F	120	2	4.69	±0.26	20.9	±1.30
	F	140					
2-R	Pool M	150	2	4.15	±0.25	21.2	±1.14
	M	145					
3-R	Pool M	135	2	5.30	±0.26	19.8	±1.20
	F	125					

TABLE 5

NO. OF MITOCHONDRIA PER GRAM OF FRESH RAT LIVER IN THE NUCLEAR (N) AND MITOCHONDRIAL (M) FRACTIONS ISOLATED IN 0.25 M SUCROSE. INFLUENCE OF THE DIET

Rat no.	Sex	Weight Before (gm.)	Weight After (gm.)	Diet	Days on diet	N fraction $\bar{X}\times10^{-10}$	N fraction A	M fraction $\bar{X}\times10^{-10}$	M fraction A
221	Pool F	150	110	Fasting	6			34.4	±1.17
	F	150	115						
232	F	150	140	Synthetic	120	2.32	±0.16	19.7	±1.20
232-A	F	154	150	"	120	3.45	±0.23	20.2	±1.10
247	Pool M	150	110	Cooked rice	90	3.69	±0.31	23.0	±1.10
	F	150	120						

TABLE 6

TOTAL NO. OF MITOCHONDRIA PER RAT LIVER CELL IN DIFFERENT PHYSIOLOGICAL AND PATHOLOGICAL CONDITIONS

	No. of mitochondria/gm of fresh liver	No. of cells/gm of fresh liver	No. of mitochondria/cell
Normal	33.0×10^{10}	133×10^{6}	2,480
Total liver with tumor	41.9×10^{10}	92×10^{6}	4,550
Pure tumor	39.4×10^{10}	554×10^{6}*	711
Regeneration 2 days	25.7×10^{10}	123×10^{6}†	2,089
Cooked rice 90 days	26.7×10^{10}	137×10^{6}	1,940

* Calculated from DNA concentrations given by Price *et al.* (11, 12).
† Taken from Price and Laird (11).

TABLE 7

COMPARISON OF REPLICATE COUNTS ON THREE DIFFERENT PRISMS BY THREE OPERATORS

Prisms	Operators a			b			c		
1	23	28	27	27	29	27	30	28	26
2	35	34	30	32	41	29	32	19	26
3	26	25	28	24	29	25	39	41	37

Operators	t	Prisms	t
a versus b:	0.3645	1 versus 2:	1.7064
a " c:	0.9000	1 " 3:	1.3884
b " c:	0.5743	2 " 3:	0.1471

t values between operators for prisms 1, 2, and 3; and t values between prisms for operator a, b, and c, where t value for significance with a probability of 0.95 is 2.120.

significant, as indicated in detail in Table 7. It shows also that there is no significant difference between prisms (Nos. 1, 2, 3).

Series of counts on replicate dilutions of the same mitochondrial fraction are statistically the same. Whether 22 or 50 prisms are counted, the same accuracy is obtained. The mean values are, respectively, 28.9 and 30.0 for 22 counts and 50 counts. The number of prisms counted has been set at 26 for convenience in the statistical analysis.

Results.—As reported in Table 1, the number of mitochondria found per gram of fresh tissue, whether the particulates are isolated in 0.25 M or 0.88 M sucrose, is the same. Furthermore, we feel that agreement between the summation of the number of particles in the (N) and (M) and (S) fractions and that of the homogenate confirms the accuracy of the results.

The mean percentage of mitochondria present in the nuclear fraction isolated in sucrose has been approximated at 10–18 per cent by enzymatic studies on particulates (17). A mean of 11 per cent is obtained by direct counting on eleven different (N) fractions isolated from liver. Accurate corrections of results in biochemical investigations on particulates seem possible if counts are made after each separation.

A correlation of the variation of the mitochondria population with the number of cells has been presented in Table 6. The number of mitochondria per cell seems much more interesting than the number per gram of fresh tissue, though both methods of expression are useful. Comparison of the number of mitochondria with the yield in grams may indicate whether the *mitochondria* are normal or abnormal, and a comparison of the number of mitochondria with the number of cells per gram may show whether the *cells* are normal or abnormal. In fact, by comparing the number of mitochondria per gram of fresh tissue with the yield of mitochondria per gram of fresh tissue, we have suggested that the mitochondria of tumor cells are smaller than normal mitochondria, since their number increases though the dry weight decreases. One may speculate on the presence of pathological mitochondria in tumor tissue.

The fact that the number of mitochondria per neoplasic liver cell seems less than in the normal or regenerating liver cell is of great importance. It remains to be shown if the phenomenon is characteristic of neoplasia. Further investigation on these topics is necessary before these variations may be accurately defined.

Since the number of mitochondria varies in different physiological and pathological conditions, it would appear more appropriate to express the concentrations or enzymatic activities of metabolites in relation to the number of mitochondria per cell when working with particulates. This extends the suggestion of Boivin and the Vendrelys (2) and of other investigators (4, 5, 11) to express biochemical phenomena in relation to the number of cells.

SUMMARY

A method has been devised for counting mitochondria obtained by centrifugation of rat liver homogenate in sucrose. Values for normal rat liver are given. Preliminary results are furnished for tumor and regenerating liver and for fasting rats. The influence of diet is also considered.

The number of mitochondria per normal rat liver cell is approximately 2,500. The mitochondrial population of the rat liver cell seems to vary in different physiological and pathological conditions.

Boivin and the Vendrelys' suggestion to express biochemical phenomena per cell is extended. When dealing with mitochondrial fractions it is proposed to express results per mitochondrion.

ACKNOWLEDGMENTS

We wish to thank Dr. A. Frappier, director of l'Institut de Microbiologie de l'Université de Montréal, for the use of the electron microscope. Our thanks are extended to Mr. A. Nantel of the same Institute for his guidance in the work with the apparatus. We thank Dr. Genest, director of the Veterinary Research Laboratories, St-Hyacinthe, Quebec, for the loan of the phase microscope.

We express our gratitude to Mr. R. Daoust of our laboratory, for cell counts and also to Mr. Y. Grandchamp, for the statistical analysis.

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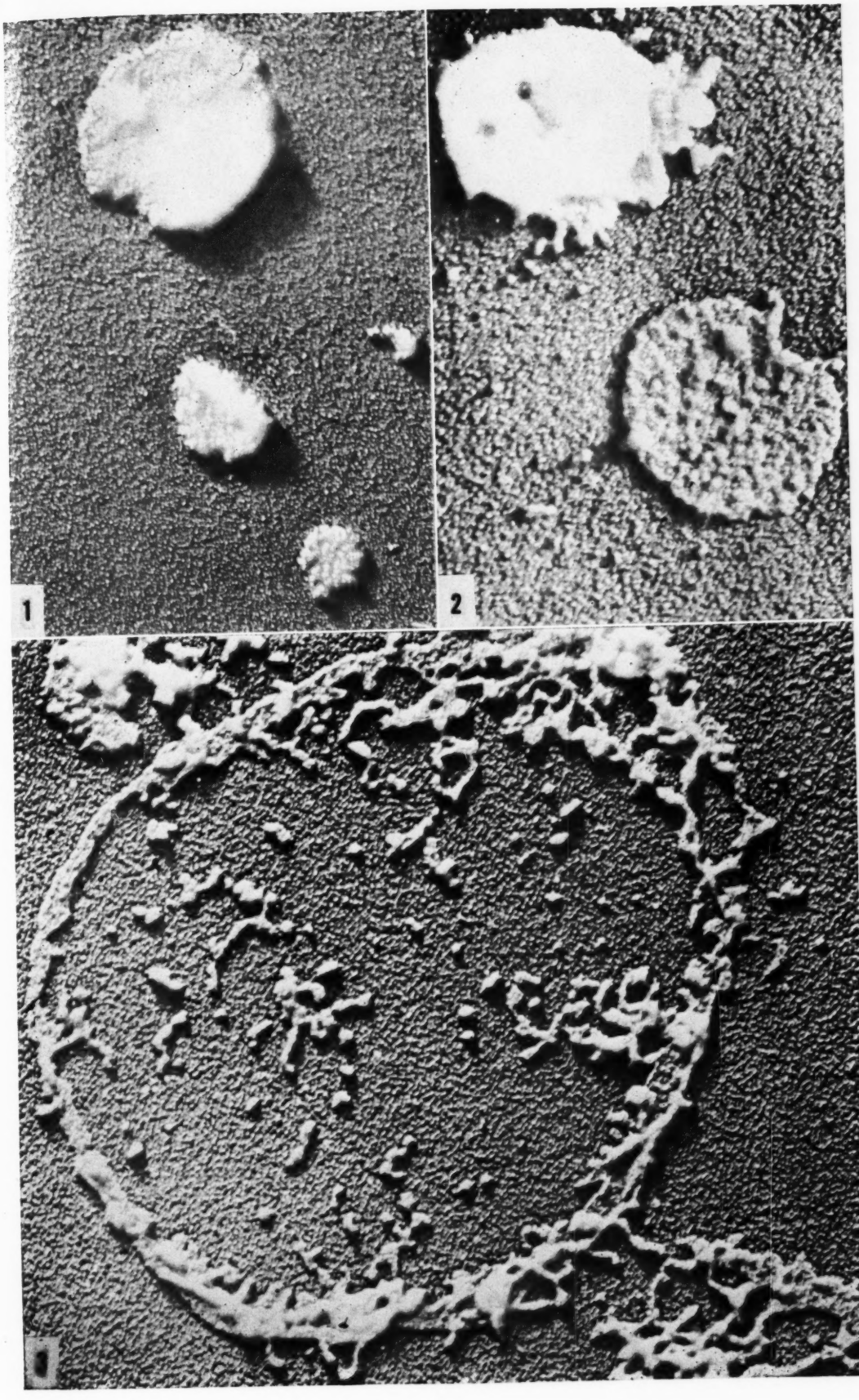
Fig. 1.—Spherical mitochondria from liver tissue isolated in 0.25 M sucrose. Electron micrograph gold shadowed without fixation. Mag. ×19,000.

Fig. 2.—Spherical mitochondria disintegrating. Electron micrograph gold shadowed without fixation. Mag. ×26,000.

Fig. 3.—A spherical mitochondrion ghost. Electron micrograph gold shadowed without fixation. Mag. ×36,000.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Effects of A-Methopterin (4-Amino-N^{10}-methylpteroylglutamic Acid) on the Synthesis of the Adenine Moiety of Adenosine Triphosphate

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It has been demonstrated that formate is a precursor of the 2- and 8-carbon atoms, glycine is a precursor of the 4- and 5-atoms of uric acid in pigeons (7), and that similar utilization of these precursors is operative in nucleic acid purines in the rat (8). Heinrich and Wilson (3) have observed that glycine, formate, and CO_2 are precursors of "nucleotides" in the rat. We have reported that folic acid antagonists will inhibit the incorporation of C^{14}-formate into nucleic acid purines (6). It seemed of interest, therefore, to determine whether formate and glycine were actually precursors of ATP adenine, and, if so, whether folic acid antagonists would likewise inhibit *in vivo* synthesis of ATP adenine from C^{14}-formate and C^{14}-glycine.

A much slower uptake of preformed adenine into ATP than into nucleic acids has been reported (2). Similarly, a very slow uptake of ammonia nitrogen into ATP adenine has been observed (1).

Knowledge of ATP synthesis appears of possible importance in the cancer field, in view of the interesting postulates made by Potter (5). It was suggested that the reason for unrestrained growth of cancer tissue might be the displacement of the synthesis of an enzyme (*X*) by the synthesis of "cancer virus," which might result in a prevention of the normal mechanism for controlling growth. This mechanism was considered to be the removal of essential building blocks by the action of the respiratory enzymes. Enzyme (*X*) was suggested to be "a constituent of normal differentiating cells and on the basis of present data is thought to be a complex of respiratory enzymes possibly of the nature of a ribonucleoprotein complex." The balance between adenosine triphosphate (ATP) and its breakdown products was considered as a possible controlling factor in tissue equilibriums related to growth.

It was the objective of the present study to carry out certain preliminary experiments which might provide a means of investigating the dynamics of ATP adenine turnover in normal and malignant tissue and the effects of anti-cancer agents on ATP adenine turnover in such tissue.

Received for publication January 5, 1952.

EXPERIMENTAL

Groups of four mice received intraperitoneal injections of 2.0 μc. each of C^{14}-formate or 5.0 μc. each of glycine-1-C^{14} and after 6 hours were sacrificed, and the whole carcasses of the animals (minus skin and hair, tails, and feet) were quickly frozen prior to ATP isolation.

TABLE 1

FORMATE AND GLYCINE INCORPORATION INTO ATP ADENINE AND THE EFFECTS OF A-METHOPTERIN THEREON

Exp. no.	Precursor	Treatment	Time of sacrifice (hours)	ATP adenine specific activity
1	C^{14}-formate	None	1	96.7
2	C^{14}-formate	A-methopterin	1	41.6
3	C^{14}-formate	None	6	91.2
4	C^{14}-formate	A-methopterin	6	70.3
5	C^{14}-glycine	None	1	33.4
6	C^{14}-glycine	A-methopterin	1	26.7
7	C^{14}-glycine	None	6	71.0
8	C^{14}-glycine	A-methopterin	6	38.4
9	C^{14}-glycine	None	24	39.7
10	C^{14}-glycine	A-methopterin	24	16.6

Note: Specific activity in μc/M C. C^{14}-formate was injected at a level of 2.0 μc/mouse, while C^{14}-glycine was given at a level of 5.0 μc/mouse. In each experiment four animals were injected with the labeled compound indicated and their carcasses pooled for ATP isolation.

The procedure for isolation of the ATP was that of LePage (4). Analysis of the barium salt of ATP isolated by this procedure indicated a purity of the order of 99 per cent. The ultraviolet spectrum was identical with that of our best commercial samples of ATP. The ATP was hydrolyzed with perchloric acid, and the adenine moiety was separated on a Dowex 50 ion-exchange column. The fact that only adenine was present in the elution pattern proves the absence of nucleic acids from the isolated ATP. The adenine concentration in the eluate was determined spectrophotometrically, carrier adenine was added, and the adenine was then

precipitated as the silver salt. The silver salt was oxidized, and the resulting barium carbonate was assayed for activity by usual procedures.

In a second series of experiments, mice were treated with 3 mg/kg of A-methopterin (4-amino-N^{10}-methylpteroylglutamic acid) per day for 2 days and were then injected with 2.0 μc. of C^{14}-formate or 5.0 μc. of glycine-1-C^{14}. Carcass ATP was isolated from these mice at 6 hours after administration of the labeled purine precursor.

The specific activities of the ATP adenine from control and A-methopterin-treated mice are presented in Table 1.

DISCUSSION

It can be seen from the control data that formate and glycine are effective precursors at ATP adenine. When these data are compared with previous data from this laboratory on formate incorporation into nucleic acid adenine[1] of mouse viscera, it can be seen that nucleic acid adenine and ATP adenine are of the same order of activity (Table 2). This is in agreement with the results of Heinrich and Wilson (3), who obtained similar data with "nucleotide" adenine and nucleic acid adenine.

TABLE 2

FORMATE INCORPORATION INTO ATP ADENINE AND NUCLEIC ACID ADENINE (AT 6 HOURS)

Source	Specific activities (μc/mol of C) Controls	A-methopterin	Per cent reduction
ATP adenine	91	70	23
DNA adenine	130	6	95
RNA adenine	125	22	83

Note: ATP adenine from mouse carcasses; DNA and RNA adenine from viscera; 2.0 μc. of C^{14}-formate used in all experiments.

[1] L. L. Bennett, Jr., H. E. Skipper, J. Hill, J. Meade, M. Brannan, and L. Simpson, unpublished data.

It is also clear from the data presented in Table 1 that A-methopterin has effectively inhibited incorporation of formate and glycine into ATP adenine. Since different tissues have been used in the studies on nucleic acids and on ATP, no strict comparisons are possible regarding the relative effects of A-methopterin on these fractions. Further studies of this nature on comparable tissues and tumors are under way.

SUMMARY

It has been demonstrated that formate and glycine are effective precursors of ATP adenine and that A-methopterin inhibits the synthesis of this nucleotide.

ACKNOWLEDGMENTS

This work was supported by grants from the American Cancer Society, upon recommendation of the Committee on Growth, the Biology and Medicine Division of the Atomic Energy Commission, and Mr. Ben May, Mobile, Ala.

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Oxidative Phosphorylation Catalyzed by Cytoplasmic Particles Isolated from Malignant Tissues

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Investigations by E. Boyland and his collaborators (2, 3) suggested that a phosphorylating glycolytic mechanism, similar to that occurring in normal mammalian cells, was present in a number of malignant tissues. Subsequent investigators (19, 21) have obtained evidence in support of this contention and have further shown (18) that unfractionated tumor homogenates are capable of a vigorous esterification of inorganic P to adenine nucleotide acceptors concomitant with the glycolysis of HDP[1] in a fluoride-containing system. Whether phosphorylations coupled with the oxidation of tricarboxylic acid cycle substrates play an important role in the synthesis of ATP in malignant tissues remains, however, a moot point. In 1945 Potter (22) observed that the addition of succinate practically prevented the large output of inorganic P which occurred when hepatoma homogenates were incubated in air in the presence of a suspension medium containing inorganic P, fluoride, and ATP, and it seems likely that phosphorylation coupled to the oxidation of succinate took place under these conditions. More recently, the oxidation of tricarboxylic acid cycle substrates and fatty acids has been shown to proceed rapidly in tumor slices (32), and pyruvate oxidation can be demonstrated in tumor homogenates if suitable precautions are taken to combat the excessive dephosphorylation of adenine nucleotides which occurs in such preparations (25). These facts, together with the demonstration that certain tumors can utilize the energy derived from succinate oxidation for biosynthetic purposes (16), strongly suggest that phosphorylative oxidations indeed play an important part in the intracellular dynamics of tumor tissue.

In this paper we shall demonstrate that cytoplasmic particles isolated from a number of transplantable tumors will catalyze an esterification of inorganic P to adenine nucleotide acceptors coupled to the oxidation of succinate. This phosphorylation cannot be accounted for in terms of glycolytic reactions and is sensitive to low concentrations of DNP. With the use of P^{32} as an indicator, it has been possible to demonstrate that inorganic P is incorporated into the phospholipid, nucleic acid, and "phosphoprotein" fractions of the tumor particles under the conditions of the succinate-linked oxidative phosphorylation. The incorporation of inorganic P into these acid-insoluble fractions is likewise sensitive to DNP and dependent upon the addition of substrate.

METHODS AND MATERIALS

Preparation of cytoplasmic particles.—The tumors were removed immediately after killing the animals, and any necrotic material and fibrous strands were dissected away. Portions of approximately 2 gm. were placed in iced tubes containing 10 ml. of 0.25 M sucrose-0.01 M $KHCO_3$. The tissue was then homogenized in a tight-fitting, all-glass homogenizer immersed in an ice bath, approximately 15 per cent homogenates being prepared. All subsequent manipulations were conducted at 0°.

"*Sucrose*" *particles.*—These were prepared by centrifuging the homogenates twice at 600 × *g* for 3 minutes, the precipitates being discarded. The supernatant fluid was spun at 14,000 × *g* for 20 minutes, and the precipitate washed twice with sucrose and then suspended in this medium. The final suspension was then centrifuged at 600 × *g* for 3 minutes, and any precipitate remaining was discarded.

"*Sucrose-KCl,*" *particles.*—These were isolated after removing nuclei and cell debris by centrifugation 2 times at 600 × *g* for 3 minutes. The supernatant fluid was then made 0.15 M with respect to KCl by the addition of appropriate volumes of 2 M KCl. The precipitate resulting from centrifu-

* Daisy Schwimmer Fellow in Cancer Research.

[1] The following abbreviations are employed: HDP = hexose diphosphate; AMP = adenosine-5-phosphate; ADP = adenosine diphosphate; ATP = adenosine triphosphate; TCA = trichloroacetic acid; TRIS = tris(hydroxymethyl)aminomethane; DNP = 2,4-dinitrophenol; DPN = diphosphopyridine nucleotide.

Received for publication January 8, 1952.

gation of this fluid at 2,000 × *g* for 30 minutes was washed twice with 0.15 M KCl and finally suspended in this medium.

Determination of P:O ratio.—This was performed according to the "interval" method described by Judah and Williams-Ashman (14). The reactions were carried out in a final volume of 3.0–3.2 ml. and were stopped by the addition of TCA. The components of the reaction mixtures are recorded under the relevant protocols.

Analytical procedures.—Oxygen uptakes were measured manometrically in conventional Warburg flasks with air as the gas phase and 0.2 ml. 10 per cent KOH and filter paper roll in the center well. The flasks were allowed to equilibrate 5 minutes before the beginning of measurement of oxygen consumption. Inorganic ortho-P was measured either by the method of Fiske and SubbaRow (10) or that of Gomori (13). Acid-labile P was determined after hydrolysis in 1 N HCl for 10 minutes at 100°. Radioactivity was measured on samples in solution or in the dry state as previously described (12).

Succinic acid and DNP were purified by recrystallization from hot water, and TRIS was purified by recrystallization from aqueous acetone. The cytochrome c was a commercial preparation containing 0.34 per cent iron, the concentration of the stock solutions being determined spectrophotometrically. P^{32} was obtained from the Atomic Energy Commission as orthophosphoric acid. This preparation was subjected to hydrolysis at 100° in 1 N HCl for 20 minutes, cooled, neutralized, and crystallized as magnesium ammonium phosphate after the addition of small amounts of carrier P^{31} ortho-P. The crystalline product was then washed with 1 N NH_4OH and brought into solution by the addition of dilute HCl. AMP, ADP, and ATP were commercial preparations, the first being obtained as the free acid and the latter two as the dibarium salts. The AMP and ADP were practically free of inorganic P, while the ATP usually contained some inorganic ortho-P, but never more than 8 per cent of the total acid-labile P. DPN was purified by counter-current distribution and was assayed enzymatically with yeast alcohol dehydrogenase (26).

Yeast hexokinase was isolated from Red Star yeast according to the procedure of Berger *et al.* (1). Partially purified preparations ("Preparation I") were carried as far as stage 3*a*, being stored in 10 per cent fructose at −25°. Purified preparations[2] ("Preparation II") were carried as far as stage 6 and were stored in 1 per cent glucose at 0°.

[2] We are greatly indebted to Dr. S. S. Barkulis for gifts of this purified hexokinase.

The tumors were obtained from multiple subcutaneous inoculations on the ventral abdominal surface,[3] with the exception of the Ehrlich ascites tumor, which was maintained by intraperitoneal inoculation of the ascitic fluid.

RESULTS

Oxidative phosphorylation with hexokinase and fructose as terminal P acceptor system.—Initial experiments were performed in which tumor particles were isolated from homogenates prepared in 0.15 M KCl by centrifugation at 2,000 × *g*, with the use of particles equivalent to approximately 250 mg. fresh tissue. Low rates of succinate oxidation occurred under these conditions, and no P uptake was observed. Fractionation of tumor homogenates prepared in 0.25 M sucrose revealed the fact that the mitochondrial precipitate from all the tumors studied was very much smaller than that derived from the same initial wet weight of liver and kidney. Exploratory investigations showed that, although oxidative phosphorylation could be demonstrated with particles isolated from homogenates prepared in 0.15 M KCl if large amounts of tissue were employed, particles isolated from sucrose homogenates were the most satisfactory type of preparation. The agglutination of such particles by the addition of KCl after the removal of nuclei and cell debris ("sucrose-KCl" particles) appeared to be the best procedure, especially since such particles can be sedimented at low speeds. For the isolation of particles capable of catalyzing oxidative phosphorylation, it was found necessary to use particles equivalent to at least 1 gm. of fresh tissue in each vessel. In the experiments described below, between 0.6 and 1.7 mg. of particle N was added to each flask.

In the presence of the various reagents necessary for oxidative phosphorylation (inorganic P, Mg^{++}, adenine nucleotide, and fluoride), negligible oxygen uptake occurred whether or not cytochrome c (0.00005 M) was added. Succinate was readily oxidized, maximal rates of oxidation being observed at a final concentration of 0.033 M. Under the conditions used for the measurement of P:O ratios, the Q_{O_2} (N) (μl O_2 consumed/mg N/hour) for succinate oxidation, catalyzed by particles isolated from sucrose homogenates, was of the order of 200 at 25°. With well washed particles, cytochrome c invariably enhanced the rate of succinate oxidation. The magnitude of the increase in succinate oxidation caused by addition of cytochrome c varied considerably from one preparation

[3] The tumor transplants were kindly maintained by Dr. Paul Talalay, Marie Dobson, George V. M. Takano, and William Bradford.

to another, even with particles isolated from the same type of tumor, increases of two- to tenfold being observed with particles from Flexner-Jobling carcinoma. Even in the presence of cytochrome c, a number of other tricarboxylic acid cycle substrates were not oxidized when added to a final concentration of 0.01 M, e.g., fumarate, glutamate, α-ketoglutarate, and citrate. The tumor particles appeared to contain negligible amounts of functionally active DPN, since the further addition of nicotinamide (0.016–0.032 M) and DPN (0.001 M) barely affected the endogenous respiration but permitted glutamate (0.01 M) to be oxidized; a typical experiment is summarized in Table 1. Fluoride, an obligatory component for the demonstration of oxidative phosphorylation, inhibited succinate and glutamate oxidation about 30 per cent at a final concentration of 0.013 M.

TABLE 1

DPN REQUIREMENT FOR GLUTAMATE OXIDATION CATALYZED BY TUMOR PARTICLES

Glutamate (μM)	Nicotinamide (μM)	DPN (μM)	KF (μM)	O_2 uptake (μatoms/30 min)
0	0	0	40	0.3
30	0	0	40	0.5
0	100	3	40	1.2
30	100	3	40	4.7
30	100	3	0	6.7

"Sucrose-KCl" particles isolated from Flexner-Jobling carcinoma. Reaction carried out in final volume of 3.0 ml. containing 11 μM K phosphate, pH 7.4; 40 μM glycylglycine, pH 7.5; 150 μM KCl; 0.15 μM cytochrome c; 2 μM ATP; 12 μM AMP; and 20 μM $MgCl_2$. Temp. 22° C.

The first experiment summarized in Table 2 shows that a marked uptake of inorganic P occurred when succinate was oxidized by washed tumor particles with hexokinase and fructose as terminal P acceptor systems. No P uptake was associated with the small oxygen consumption which occurred in the absence of added substrate, and the succinate-linked phosphorylation was depressed by DNP to a much greater extent than the oxidation of succinate per se. Other experiments showed that the same picture was observed with either type of hexokinase preparation. It may be remarked that "Preparation I" hexokinase was not completely free of certain other glycolytic enzymes, while "Preparation II" was completely so; the addition of either type of preparation and sugar barely affected the succinate oxidation. These facts indicate that the phosphorylation taking place under these conditions cannot be accounted for in terms of glycolytic reactions and is similar to that observed with mitochondria isolated from either liver or kidney.

"Sucrose" particles isolated from Flexner-Jobling carcinoma lose their ability to catalyze oxidative phosphorylation if incubated at 25° in the absence of added substrate. Thus, after 37 minutes of incubation the rate of succinate oxidation was depressed only 20 per cent, while the P:O ratio was diminished by 74 per cent.

With hexokinase-fructose as terminal P acceptor, phosphorylation linked to succinate oxidation was demonstrated with particles from Flexner-Jobling carcinoma, Jensen sarcoma, Walker 256 carcinoma, a mouse amelanotic melanoma, typical experiments being summarized in Table 3. Similar experiments with particles isolated from the Ehrlich ascites tumor have been recorded elsewhere (17). P:O ratios in excess of unity were observed in a sufficient number of experiments to justify the tentative conclusion that the "true" P:O ratio for succinate oxidation under these conditions is 2.

Oxidative phosphorylation with ADP as terminal P acceptor system.—Further proof that the P esterification associated with succinate oxidation was a reflection of aerobic phosphorylations was afforded by experiments in which ADP was substituted for hexokinase and fructose as the terminal P acceptor. Under these conditions the possibility of contaminating glycolytic reactions is even less likely. It can be seen from the second experiment summarized in Table 2 that, with ADP as acceptor, tumor particles catalyze an uptake of inorganic P which was dependent upon the addition of succinate and inhibited by DNP. However, the P:O ratios observed with ADP as a P acceptor system were invariably lower than those found with hexokinase and fructose. A few experiments showed that AMP (8 μM) and ATP (2 μM) mixtures were even less satisfactory as a phosphate acceptor system.

TABLE 2

OXIDATIVE PHOSPHORYLATION CATALYZED BY PARTICLES ISOLATED FROM FLEXNER-JOBLING CARCINOMA

Exp.	Phosphate acceptor system	Additions	ΔP (μM)	ΔO_2 (μatoms)	P:O
1	0.03 ml. hexokinase (Preparation II); 111 μM D-fructose; 5 μM ATP	Nil	+0.2	0.6	
		Succinate	−7.9	12.1	0.66
		Succinate +DNP	+1.0	9.5	
2	10 μM ADP	Nil	+0.3	0.0	
		Succinate	−2.5	12.0	0.21
		Succinate +DNP	+1.6	11.0	

Each flask contained 10 μM K phosphate, pH 7.4; 50 μM glycylglycine, pH 7.4; 0.1 μM cytochorome c; 60 μM KF; 20 μM $MgCl_2$; 1.0 ml. "Sucrose-KCl" particles (150 μM KCl). If added: 100 μM succinate; 0.15 μM DNP. Forty minutes incubation at 27° in both experiments.

Tracer experiments with radioactive P.—Friedkin and Lehninger (12) have shown that isolated mitochondria from normal liver tissue are not only capable of incorporation of radioactive inorganic P into the pyrophosphate linkages of ATP, but are

also able to carry out a number of important synthetic reactions involving the incorporation of P^{32} into the acid-insoluble fractions of these particles. These workers found that, when oxidative phosphorylation occurred in the presence of radioactive inorganic P, the phospholipid, nucleic acid, and "phosphoprotein" fractions rapidly became radioactive and presented convincing evidence that this incorporation of radioactivity was dependent upon oxidative phosphorylation. In view of the interest which is inherent in the problem of how tumor cells carry out such vital synthetic reactions, it was considered valuable to determine whether the same synthetic mechanisms could be found in particles obtained from Flexner-Jobling carcinoma.

In these experiments "sucrose" particles were used as a source of enzyme, and the conditions were similar to those employed for the determination of P:O ratios. An exact description of the experimental conditions during incubation is given in Table 5. The experiments were terminated by the addition of 2.0 ml. of 10 per cent TCA and the contents of the vessels then subjected to fractionation based on the schemes used by Friedkin and Lehninger (12) and by Schneider (27). This procedure was essentially the following:

1. Centrifugation of the suspensions and repeated washing of the precipitate with 5 per cent TCA. The combined supernatant and washings constituted the acid-soluble fraction.

2. The residue from Step 1 was then repeatedly extracted with ethanol, as described by Schneider (27). The ethanol extracts were combined and represented the phospholipid fraction.

3. The residue from Step 2 was then extracted at 100° for 15 minutes with 5 per cent TCA, centrifuged after cooling, and the residue repeatedly washed with 5 per cent TCA. The combined extract and washes constituted the nucleic acid fraction.

4. The washed residue from Step 3 constituted the "phosphoprotein" fraction.

The fractions so obtained may be grossly contaminated with inorganic P^{32} which is adsorbed on the TCA precipitates (12). The phospholipid fraction was freed of contaminating inorganic P^{32} as follows: 5.0 ml. of the ethanol extracts was pipetted into a glass-stoppered vessel, and 10.0 ml. of ethyl acetate was added. To this solution, 3.0 ml. of a solution of 0.5 M $Na_2HP^{31}O_4$ containing 2.0 M KCl was added. The two phases were equilibrated by inverting 100 times, and the aqueous phase was removed with a capillary syringe. The organic phase was then washed twice more in exactly the same fashion. By this treatment the organic phase, containing the phospholipid, was completely freed of contaminating inorganic P^{32}.

The extract of nucleic acid (Step 3) was freed of contaminating inorganic P^{32} by the method of Ernster *et al.* (8), which has proved very effective in this work, particularly if the extraction with isobutyl alcohol–benzene was repeated after the addition of carrier inorganic P^{31}.

The efficiency with which contaminating inorganic P^{32} was removed from these fractions was indicated by a "zero time control" to which TCA was added at the beginning of the incubation and then carried through the entire fractionation procedure.

It was found that the phosphorus distribution in particles isolated from Flexner-Jobling carci-

TABLE 3

OXIDATIVE PHOSPHORYLATION IN TRANSPLANTABLE TUMORS

Exp.	Tumor	Particle prep.	Inorganic phosphate added (μM)	Temp. (°)	Substrate	ΔP (μM)	ΔO_2 (μatoms)	P:O
1	Amelanotic melanoma (mouse)	"Sucrose" (1.6 mg. N)	23	21	Nil	+ 2.5	1.5	
					Succinate	−19.9	16.0	1.24
2	Walker 256 carcinoma (rat)	"Sucrose-KCl"	10	27	Nil	+ 1.2	1.9	
					Succinate	− 4.2	13.1	0.32
3	Jensen sarcoma (rat)	"Sucrose" (1.3 mg. N)	10	22	Succinate	− 6.2	10.1	0.61

Conditions as in Experiment 1 of Table 2, except that "Preparation I" hexokinase (0.2 ml.) was used in each case; 40 minutes' incubation.

TABLE 4

DISTRIBUTION OF P IN CYTOPLASMIC GRANULES DERIVED FROM FLEXNER-JOBLING CARCINOMA

Fraction	P content (μg.)	Per cent Total P
Total	106	(100)
Phospholipid	43	40
Nucleic acid	27	25
"Phosphoprotein"	4	4

The method of fractionation of TCA extracts of the granules is described in the text. Particles contained 0.67 mg. N.

noma was similar to that observed in previous work with normal liver mitochondria (12). Data from a typical experiment are given in Table 4.

Several experiments with Flexner-Jobling particles presented essentially the same picture. Extensive incorporation of P^{32} into the acid-insoluble fractions by mechanisms requiring the presence of added substrate and sensitive to the action of DNP was observed in every experiment. Results of two typical experiments are given in Table 5.

TABLE 5

INCORPORATION OF RADIOACTIVE INORGANIC P INTO ACID-INSOLUBLE FRACTIONS OF TUMOR CYTOPLASMIC GRANULES

	Specific Activity (Counts/μg P/min)					
	Phospholipid		Nucleic Acid		"Phosphoprotein"	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
Complete system	387	48	725	64	3,410	985
Substrate omitted	60	5	122	15	—	104
DNP added	24	3	114	9	196	59
Zero time control	0	0	11	0	64	45

The experiments were run in Warburg vessels at a temperature of 30° under an atmosphere of air and were terminated after 20 minutes. Contents of the vessels for the complete system were as follows: $MgCl_2$, 15 μM; ATP, 3.0 μM; cytochrome c, 0.5 mg.; succinate, 100 μM; NaF, 40 μM; TRIS buffer, pH 7.4, 50 μM; KCl, 150 μM. 0.10 Ml. of a solution of P^{32} was added to each vessel; approximately 30 μc. and 6 μc. being added in Experiments 1 and 2 respectively. Water was added to bring the volume to 2.0 ml., and 1.0 ml. of a sucrose suspension of cytoplasmic particles from Flexner-Jobling carcinoma was added immediately before incubation. Succinate was omitted in one set of vessels, and 1.0 μM of 2,4-dinitrophenol was added to another pair, as shown in the table above. "Zero time controls" were treated identically with the complete system, except that 2.0 ml. of 10 per cent TCA were added at the beginning of the experiment rather than at the end. Fractionation procedure described in the text was used for all vessels.

It can be seen from these results that extensive incorporation has taken place into all the acid-insoluble fractions. As in earlier investigations with normal liver particles (12), the specific activities of the nucleic acid and "phosphoprotein" fractions are relatively high in relation to that of the phospholipid. Omission of substrate or addition of DNP reduces the incorporation to very low levels. It may be concluded from these results that enzymatic processes capable of utilizing the energy obtained by oxidative phosphorylation for the synthesis of these essential cell substances are present in these tumor cytoplasmic granules, which are thus shown to be closely similar to mitochondria obtained from normal liver tissue.

DISCUSSION

Since the discovery of the relatively high glycolysis of tumor slices by Warburg (29), considerable speculation has been entertained concerning the relative contribution of the anaerobic and aerobic phases of carbohydrate breakdown to the supply of energy for tumor growth. The apparently low content of certain respiratory enzymes (23, 28), co-enzymes (30), and cytochrome c (7) in malignant tissues suggested that some disturbance in the oxidative capacities of tumors may exist. Moreover, the lack of accumulation of citrate in tumor tissue following the administration of fluoroacetate *in vivo* (24) and the failure of tumor homogenates to oxidize oxaloacetate under conditions propitious for the oxidation of this substrate by liver and kidney (25) have suggested that the tricarboxylic acid cycle might be deficient in or even absent from malignant tumors.

However, the far from inconsiderable respiration of tumor slices (29) and the high concentrations of citrate in many tumors (6) were obstacles to the belief that tumors were primarily dependent upon glycolytic mechanisms for the maintenance of function. In recent years reports from a number of laboratories have emphasized that tumors not only contain many of the enzymes involved in the tricarboxylic acid cycle but also that the activity of some of these catalysts, provided they are estimated by suitable methods, is of the same order of magnitude as that in normal, actively metabolizing tissues (31). Furthermore, with the aid of isotopically labeled substrates it has been possible to demonstrate that intact tumor cells burn various intermediates of hexose catabolism and also fatty acids at rates which fall within the range shown by normal tissues (32). The further use of specific enzyme inhibitors in such studies has provided strong evidence that in tumors the pathway involved in such oxidations is indeed the tricarboxylic acid cycle.

Our experiments clearly demonstrate that another aerobic process of prime importance, i.e., the generation of energy rich phosphate bonds coincident to succinate oxidation, takes place in tumors by mechanisms apparently indistinguishable from those occurring in mammalian liver. The sensitivity to DNP of such phosphorylations catalyzed by tumor particles and their dependence upon the addition of substrate exclude the possibility, suggested by Clowes and Keltch (5), that glycolytic mechanisms are responsible for such changes. The synthetic reactions carried out by tumor particles, i.e., the incorporation of P^{32} into the phospholipid, nucleic acid, and "phosphoprotein" fractions of the acid-insoluble residue, which necessitate the occurrence of oxidative phosphorylation, are likewise sensitive to DNP and omission of substrate.

The far greater efficiency of oxidative as compared to anaerobic glycolytic reactions in supporting protein synthesis (9, 16) and concentration of amino acids (4) by intact tumor cells, and the sensitivity of these processes to DNP (4, 11), strongly imply that aerobic phosphorylations, pre-

sumably of the type described above, are of cardinal importance for tumor biosyntheses.

It cannot be denied, however, that the prima facie differences in the oxidative behavior of tumor particles from that of similar preparations isolated from liver and kidney may well represent characteristics truly peculiar to malignant tissue. Thus, the ferocious dephosphorylation of adenine nucleotides under the conditions of our experiments, which disallowed even a maintenance of inorganic P concentration by a phosphorylating system in the absence of fluoride and hexokinase-fructose, is in marked contradistinction to the apparent lack of dephosphorylation of adenine nucleotides by fresh "sucrose" particles from liver (15). Again, the apparent absence of DPN and TPN from fresh tumor particles, as evidenced by the lack of oxidation of substrates whose respective dehydrogenases requires these coenzymes, is another striking difference. It may be noted, however, that a non-malignant tissue, lactating mammary gland, yields particles similarly devoid of functionally active pyridine nucleotides yet capable of vigorous succinate oxidation (20). Whether tumor particles contain "bound" pyridine nucleotides in the intact cell cannot be ascertained at present. But it may be pertinent to mention that the particles we prepared exhibited intense "DPN-ase" activity, which could conceivably be active during the course of preparation. Particles containing 1 mg. N will destroy 2–5 μM DPN per hour at 22° and pH 7.4; the coenzyme is attacked mainly at the nicotinamide-ribose linkage, since the disappearance of DPN (measured enzymatically) is approximately equivalent to the formation of free nicotinamide.[4]

The fact that fumarate is not oxidized under conditions suitable for succinate-linked phosphorylation by tumor particles suggests that such preparations may be most suitable for the study of the mechanism of the phosphorylation associated with the one-step oxidation of succinate to fumarate.

SUMMARY

Phosphorylation coupled to the oxidation of succinate has been demonstrated by the use of cytoplasmic particles isolated from a number of transplantable tumors. The phosphorylation is dependent upon the presence of substrate and is inhibited by low concentrations of 2,4-dinitrophenol. Under conditions propitious for oxidative phosphorylation by such particles, extensive incorporation of radioactive P into the phospholipid, nucleic acid, and "phosphoprotein" fractions of the particles occurs. These synthetic reactions are also dependent upon the addition of substrate and sensitive to dinitrophenol.

[4] Unpublished observations of G. Burnett and H. G. Williams-Ashman.

ACKNOWLEDGMENTS

We are indebted to Professor Albert L. Lehninger for his interest in these investigations and to Sylvia Wagner Smith for technical assistance. This work was supported by contributions from the Daisy Schwimmer Fund and by research grants from the American Cancer Society (on recommendation by the Committee on Growth of the National Research Council) and the Nutrition Foundation.

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Growth of Free Tumor Cells in the Pleural Exudate and Their Implantation into the Pleura of the Mouse*

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It is well known in the clinical laboratory (5, 6, 8, 9, 11, 13, 15) that pleural exudate from patients with thoracic tumors may contain free tumor cells, some of which show mitotic figures. The study of the diagnostic significance of these cells has long been a part of exfoliative cytology (1, 10). However, our extensive search in the literature on cancer did not reveal any mention of experiments concerning the growth potentialities of free tumor cells floating in the pleural exudate. Our previous work on the growth characteristics of free tumor cells in the peritoneal fluid (2, 3) has shown that any mouse tumor cells (Sarcoma 37, malignant lymphoma, Sarcoma 180[1], carcinoma in C3H mice,[1] carcinoma in CAF_1 mice,[1] melanoma in DBA mice,[1] etc.), inoculated into the peritoneal cavity induced an accumulation of fluid and multiplied in this medium at a high rate as free cells. Eventually, they settled in subserous connective tissue (where the serosa was damaged by the injecting needle or by injected material) and grew into solid tissue tumors. In a new series of experiments based on the same technic and method, we have found that similar phenomena of free cell multiplication in the fluid and their localization in tissues, with subsequent tumor growth, can be obtained also in another serous cavity of the mouse—the pleural cavity. However, the anatomical and histological structure of the pleural cavity determined some essential differences in growth characteristics of intrapleurally inoculated tumor cells as compared to those in the peritoneal exudate. This comparative study was found useful for analysis of experimental data reported below.

MATERIAL AND METHODS

Tumors and mice.—Sarcoma 180 was grown in strain C mice; Sarcoma 37 in CFW; carcinoma in C3H; Cloudman's melanoma in DBA 1; Ak_4 (lymphatic leukemia) in Akm; and C1498 (myelogenous leukemia) in C57 black mice.[2] For the most part, male mice of about 25 gm. in weight were selected. At least 100 mice (ten groups each of ten mice) were used for the study of each strain of tumors.

Preparation of tumor cell suspension and cell counts.—Requisite doses of tumor cells were obtained by suspending mashed subcutaneous implants in 0.85 per cent NaCl or by diluting peritoneal fluid containing tumor cells. The standard dose for inoculation was 1,000,000–10,000,000 cells, suspended in 0.2–0.4 cc. of diluted fluid. The technic of preparation of these suspensions and of cell counts was described elsewhere (2).

Inoculation of cell suspensions and withdrawal of pleural exudate.—Puncture of most areas of the thoracic wall will produce lethal hemorrhage from the surface of the lung. However, it is quite safe to puncture the phrenico-costal sinuses (which are not occupied by the lung). We preferred for this purpose a point on the right thoracic wall at the intersection of the posterior axillary line and the lowest intercostal space. Thus, all inoculations were performed in the right pleural cavity slightly above the diaphragm.

Autopsy for studying in situ *the topography of tumors inside the thorax.*—It was found most convenient to cut first through the abdominal wall and the peritoneum and, after displacing the liver and the gut downward, to excise the diaphragm, to lift the lower end of the sternum, and to pin it below the head of the mouse. Thus, both pleural cavities and the whole mediastinum are open for inspection.[3]

* This work was carried out under Contract At-(40-1)-269 with the Division of Biology and Medicine, United States Atomic Energy Commission.

[1] H. Goldie, unpublished experiments.

Received for publication January 14, 1952.

[2] Tumor strains S-180, Cloudman's melanoma, and H1494, and mice of C, C3H, DBA, and C57 black strains were obtained from the Jackson Memorial Laboratories, Bar Harbor, Maine; CFW and Akm mice, from Carworth Farms, New City, N.Y.; carcinoma in C3H and S-37 strain from the National Cancer Institute, Bethesda, Md., and Ak_4 strain of leukemia from Dr. Howard Skipper, Southern Research Institute, Birmingham, Ala.

[3] Mr. Wendell Gillett, medical photographer at Vanderbilt Medical School, prepared the photographs for this publication.

RESULTS

Accumulation of exudate.—In 77–100 per cent of mice inoculated intrapleurally with the standard dose of tumor cells, an amount of approximately 0.2–0.8 cc. of exudate could be withdrawn from the right pleural cavity after 4 or 5 days. No fluid could be obtained from melanoma-inoculated mice after 7 or 8 days, but in all groups with other tumor strains the amount of exudate remained stationary, and increased rapidly only during 1 or 2 days preceding the death of the animal. As a rule, the fluid was slightly cloudy and free of blood unless the death was due to a hemorrhage, but it was slightly hemorrhagic in nearly all carcinoma-inoculated mice.

Cellular composition of the pleural exudate.—In stained smears from the pleural exudate, the overwhelming majority of cells were obviously tumor cells (Figs. 1–8). Various stages of mitoses were found in 10–25 per cent of these cells (in the melanoma group, only 4–7 per cent). The smears showed only a small proportion of leukocytes, mostly polymorphonuclear leukocytes. This impression was confirmed by differential counts carried out in several groups each of twenty mice. Each group was inoculated with a different tumor strain. The exudate was withdrawn 4–5 (first count) or 7–8 days (second count) after inoculation. The results are shown in Table 1.

As a rule most of the leukocytes at the first count were polymorphonuclears, and a decrease in their number in the later stage was paralleled by an increase in the percentage of tumor cells. Only in the exudate produced by Ak_4 or C1498 cells were the monocytes more numerous than polymorphonuclears.

Serial transfers of tumor cells in pleural exudate.—Pleural exudate was withdrawn from five mice on the eighth or ninth day after their inoculation with a standard dose of S-180. The samples of exudate were pooled and mixed with 0.85 per cent NaCl solution in a proportion of 1:4. Doses of 0.5 cc. of this suspension were injected intrapleurally into five new mice. After 8 or 9 days their pleural cavity contained not less than 0.3 cc. of exudate with numerous tumor cells. Samples of this exudate were pooled, diluted, and injected into five new mice, as above. At least five successive transfers were performed with S-180, and similar transfers were carried out in the same way with S-37 and with carcinoma in C3H. At each transfer the examination of stained smears from withdrawn exudate revealed the predominance of tumor cells over leukocytes and the presence of mitotic figures in a considerable (not less than 10 per cent) proportion of tumor cells. Thus, several tumor strains could be grown serially in the pleural exudate as almost pure cultures of free cells.

Growth of tumor implants in the pleural cavity.—Seven or 8 days after the inoculation of S-37, S-180, carcinoma, or C1498 into the right pleural cavity, sizable tumors were found on the right mediastinal pleura. They grew in all mice into the superior or the inferior mediastinum, just above the heart or between the heart and the diaphragm. The pleural growth of S-180 or C1498 remained, as a rule, circumscribed either in the superior or (less frequently) in the inferior mediastinum (Figs. 15 and 16). However, S-37 and carcinoma in C3H, while presenting similar initial growth, encircled the heart (Figs. 9 and 11) and spread on the inferior lobe of the right lung and the surface of the diaphragm, so that the entire mediastinum was filled with tumor tissue (Figs. 10 and 12). The growth was interrupted only by the death of the animal. In four mice with intrapleural S-37 that lived longer than 30 days and were sacrificed after 31 or 40 days, the autopsy revealed large intrapleural tumors growing through the thoracic wall into subcutaneous tissue or spreading through the diaphragm retroperitoneally into the abdomen (Fig. 10). Intrapleurally injected melanoma cells induced, after 10–14 days, in 85 per cent of mice scattered, small, perfectly round (1–3 mm. in diameter) black shiny tumors over all the surfaces of mediastinal, diaphragmatic, and lower part of costal pleura; but only those localized in the mediastinum grew within the next 2 or 3 weeks into large tumors, which filled most of the right pleural cavity and eventually grew through the thoracic wall into subcutaneous tissue and spread retroperitoneally into the abdomen (Figs. 13 and 14). It should be emphasized that tumor cells of all our strains very rarely (in the melanoma, as stated above) became localized on the costal pleura. At the site of inoculation in the phrenico-costal sinuses, only a slight growth of tumor tissue, eventually regressing, was

TABLE 1

PERCENTAGE OF TUMOR CELLS IN THE PLEURAL EXUDATE AT VARIOUS INTERVALS AFTER INTRAPLEURAL INOCULATION OF A STANDARD DOSE OF TUMOR CELLS*

Tumor strain	First count	Second count
S-180	68(48–78)	82(72–89)
S-37	56(41–76)	84(79–90)
Carcinoma in C3H	57(48–62)	66(52–73)
Melanoma	53(33–89)	no exudate in the pleural cavity
Ak_4	67(57–74)	the majority of mice were dead
C1498	69(54–82)	87(72–92)

* The data show for each tumor strain and for each count the average percentage for 20 mice and (in parentheses) the range.

noted. In the case of intrapleurally injected Ak_4 cells, this was their only localization as implants in the pleura.

Survival of tumor-bearing mice.—The intrapleural tumor growth invariably induced death of the animal, either by hemorrhage and by interference with vital organs (S-37, S-180, carcinoma, melanoma) or by penetration of tumor cells into the blood stream with systemic lethal effect (Ak_4, C1498). The rate of survival at different intervals after inoculation varied with the strain of tumor, as shown in Table 2.

TABLE 2

SURVIVAL OF MICE AFTER INTRAPLEURAL INOCULATION OF 1 TO 10 MILLION TUMOR CELLS

Strain of tumor	Survival in days*
S-37	12(7–40)
S-180	15(10–22)
Carcinoma in C3H	13(8–18)
Ak_4	6(5–9)
C1498	12(9–14)
Melanoma	45(32–72)

* Average and range.

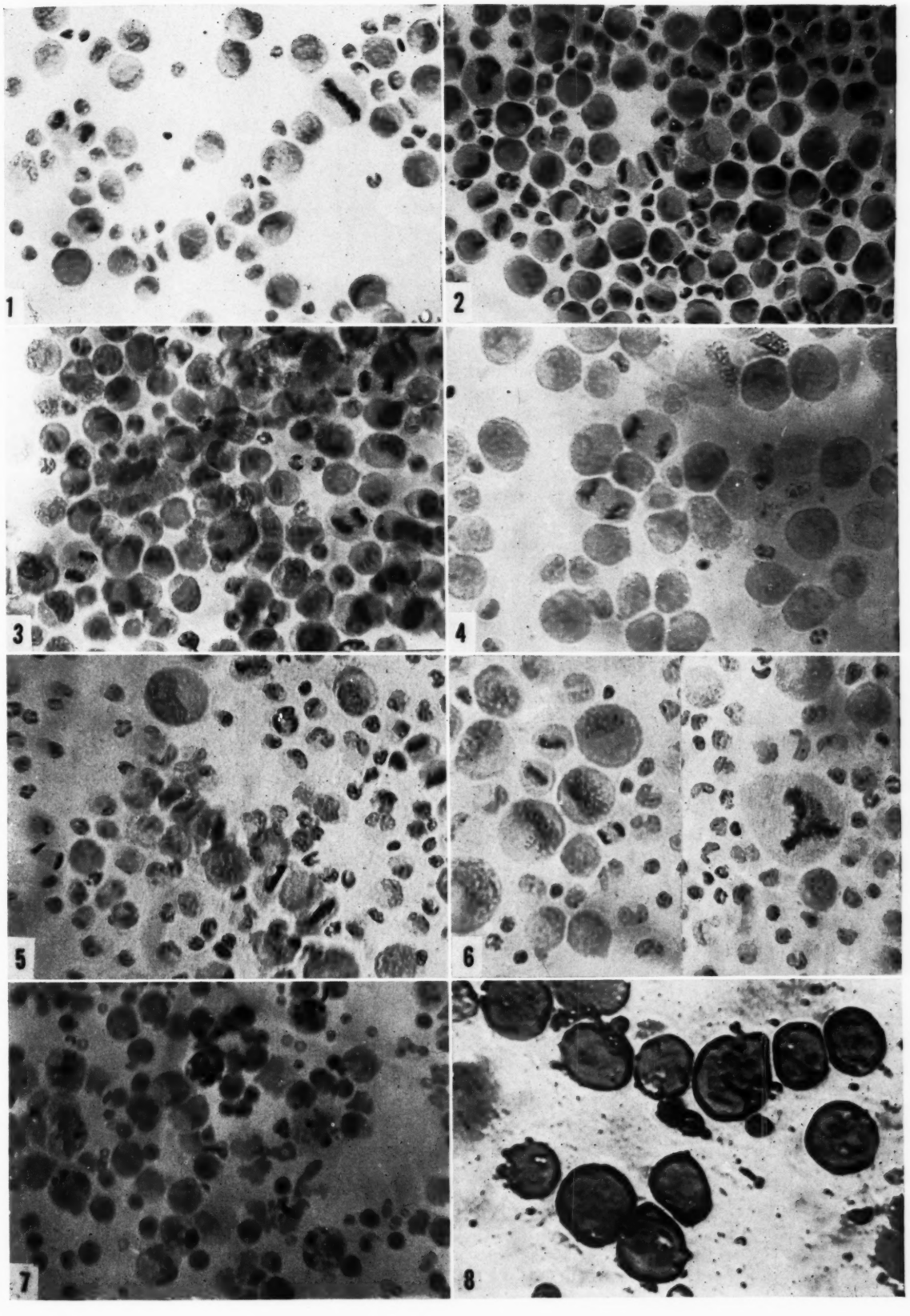
It should be mentioned that, in eight groups each of ten mice inoculated with S-37 cells from serial transfers in peritoneal fluid, none lived longer than 15 days, while out of twenty mice inoculated with cells from mashed subcutaneous implants seven lived longer than 15 days and four of them longer than 30 days. The data derived at autopsy of the latter animals were quoted above.

DISCUSSION

Students of the growth of free tumor cells in the peritoneal fluid have generally believed that this phenomenon is specific for only one tumor strain (Ehrlich's adenocarcinoma in mice—see references in [2]—or Yoshida's sarcoma in rats [14]) and occurs only in the peritoneal cavity (hence the term "ascites tumor," introduced by Loewenthal and quite recently defended by G. Klein [7]). Shields Warren and O. Gates (12) were the first to introduce a more general concept of this phenomenon by demonstrating that suspended cells of a rat tumor selected at random (Walker carcinosarcoma) grew as free cells in the peritoneal fluid; moreover, these free cells remained viable after introduction into the blood stream and induced localized tumor growth in various organs. Similarly, our previous work (2, 3,[1]) has shown that cells from any mouse tumor strain (S-37, malignant lymphoma, S-180, melanoma, etc.) can be grown in serial transfers in the peritoneal fluid of the mouse, while analogous experiments with normal mouse tissue or embryonic tissue failed completely. It was concluded that the ability to grow as a free cell in a serous fluid is a specific property of the tumor cell probably related to its autonomy. In our present work we have advanced the evidence that malignant cells of various tumor strains can be serially grown in almost pure culture in the pleural exudate of the mouse. These results indicate that the "ascites tumors" are only a special case of a more general phenomenon of free tumor cell growth in body fluids. Another special case of free tumor cells is leukemic cells which, in one stage of their life cycle, live and multiply in the blood and, in experimental conditions in the mouse, also in the peritoneal fluid (4). It may be presumed that free tumor cells may live also in other serous fluid, in tissue interspaces, etc., and that this phenomenon is an important factor in the mechanism of tumor cell growth and of the spreading tendency of tumors.

In normal mice the peritoneal cavity contains sizable amounts (0.2–0.5 cc.) of cellular fluid (2), while none is found in the pleural cavity. Moreover, inoculation of a suspension of tumor cells induced less copious accumulation of exudate in the pleural cavity than in the peritoneal cavity. These differences may be accounted for partly by the considerably greater surface area of the peritoneum and partly by its more abundant lymph drainage. The latter condition, as well as the abundance of macrophages in the peritoneum, is reflected also by large numbers of lymphocytes and macrophages in the normal peritoneal fluid and in the exudate induced by tumor cells (2), as compared to the pleural exudate, where the initial invasion by polymorphonuclear leukocytes is followed by tumor cell growth in almost pure culture.

Intrapleurally inoculated tumor cells settled in the pleural cavity and grew into solid tumors in all mice on the mediastinal pleura spreading into the mediastinum (with the exception of early melanoma and Ak_4). Thus, the tendency of free tumor cells in the peritoneal cavity to become localized, in the majority of cases, in the abdominal peritoneum (at the site of puncture), and only in the minority in the visceral peritoneum, was reversed in the pleural cavity. The interpretation of this difference should be sought, probably, in the unfavorable condition for nutrition and vascularization of tumor tissue in costal pleura, in particular in phrenico-costal sinuses, and in the favorable condition in the mediastinum which is abundantly supplied with lymphatics and blood vessels. It appears that only vigorously growing and highly vascularized mediastinal S-37 and melanoma tumors are able to spread through the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FIGS. 1–8.—Tumor cells of various tumor strains in the pleural exudate at various intervals after intrapleural inoculation.

FIG. 1.—Sarcoma 180, 5th day after inoculation. Aceto-orcein, ×600.

FIG. 2.—Sarcoma 180, 9th day. Aceto-orcein, ×600.

FIG. 3.—Sarcoma 37, 4th day. Aceto-orcein, ×600.

FIG. 4.—Sarcoma 37, 8th day. Aceto-orcein, ×600.

FIG. 5.—Carcinoma in C3H, 5th day. Aceto-orcein, ×600.

FIG. 6.—Carcinoma in C3H, 9th day. Aceto-orcein, ×600.

FIG. 7.—Melanoma, 5th day. Wright's stain, ×600.

FIG. 8.—H1498 (myelogenous leukemia). Wright's stain, ×600.

FIGS. 9–16.—Tumors of various strains grown in the pleural cavity after intrapleural inoculation of tumor cells.

FIG. 9.—Sarcoma 37, 12 days after inoculation.

FIG. 10.—Sarcoma 37, 31 days.

FIG. 11.—Carcinoma in C3H, 12 days.

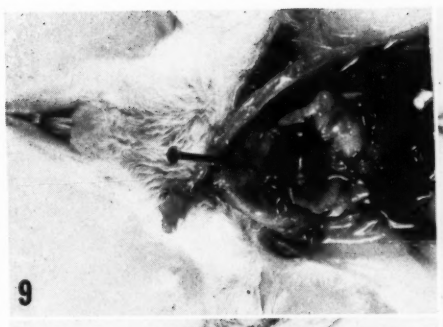
FIG. 12.—Carcinoma in C3H, 15 days.

FIG. 13.—Melanoma, 30 days.

FIG. 14.—Melanoma, 45 days.

FIG. 15.—Sarcoma 180, 9 days.

FIG. 16.—H1494, 10 days.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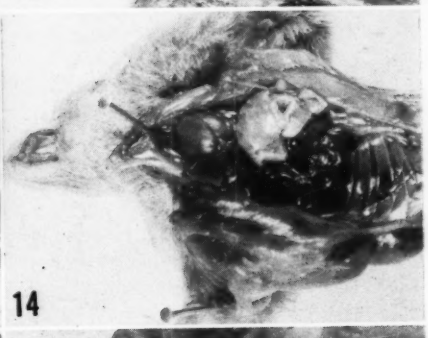
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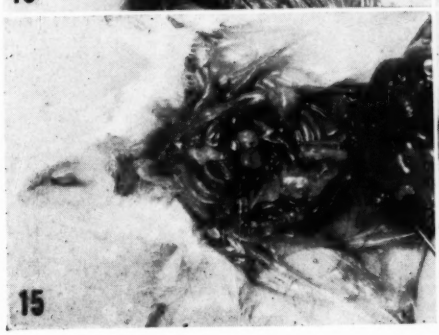
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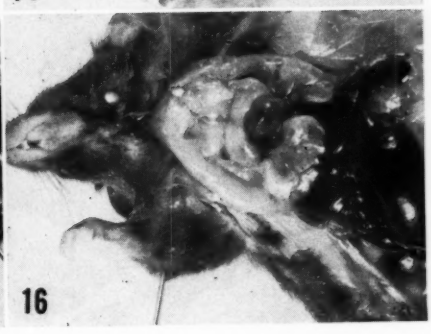
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costal pleura and the thoracic wall and establish contact with another favorable ground for growth —subcutaneous tissue. Still, it remains to be explained why these vigorous mediastinal tumors overgrew the pleural cavity where the tumor cells were inoculated but spread only very seldom into the opposite cavity.

Thus, the above reported experiments with transferable tumors in mice may provide some suggestions for interpretation of growth characteristics of spontaneous tumors in the pleural cavity. Moreover, they may serve to build up a method for therapeutic assays of physical and chemical agents on intrapleural or mediastinal tumors of mice. We are studying the effect of intrapleurally injected radioactive colloidal gold on free tumor cells and solid implants in the pleural cavity, and the results will be reported separately.

SUMMARY AND CONCLUSIONS

1. Intrapleurally inoculated suspension of tumor cells (strains S-37, S-180, carcinoma in C3H, Cloudman's melanoma, Ak_4, and C1498) induced in the pleural cavity an accumulation of exudate containing numerous tumor cells, many of them showing mitoses. The proportion of leukocytes (mainly polymorphonuclears) was significant early (4–5 days) after inoculation, but always lower than the proportion of tumor cells. In the later stage (7–8 days), these cells grew in the pleural exudate almost in pure culture. A standard dose of 1,000,000–10,000,000 cells either from mashed subcutaneous implants or from serial transfers in the peritoneal fluid was used throughout.

2. Small doses of exudate transferred into the pleural cavity of new mice induced there an accumulation of exudate with numerous tumor cells. At least five successive transfers were carried out serially with S-37, S-180, and carcinoma.

3. In each mouse (with the exception of those with Ak_4) the growth of free tumor cells in the exudate was followed by their implantation in the mediastinal pleura. They grew through the pleura into the superior or the inferior mediastinum, but, in some cases of S-37 and even more frequently in melanoma cases, they filled out most of the inoculated (right) pleural cavity and grew through the thorax wall into subcutaneous tissue where they grew luxuriantly.

4. It is concluded that the growth of free tumor cells in the pleural exudate, as well as their growth in the peritoneal fluid (ascites tumors), and the growth of leukemic cells in the blood and the peritoneal fluid are special examples of a general phenomenon of free tumor cell growth in body fluids.

5. It is hoped that the data reported above may serve for chemotherapeutic and radiotherapeutic assays on free tumor cells in the pleural exudate and on solid tumors in the mediastinum or in the pleural cavity.

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The Effect of Neoplasia on the Turnover of Nucleic Acids Studied with Formate-C^{14} and Glycine-2-C^{14}

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In three earlier papers (2, 3, 5), the authors reported an increased rate of incorporation of P^{32}-labeled sodium phosphate into the desoxyribonucleic acid (DNA) of livers and spleens in mice and rats bearing transplanted tumors. The same increase was not observed in liver nuclear pentosenucleic acid (nPNA) or cytoplasmic pentosenucleic acid (cPNA) (5). This would indicate that the increase found in the DNA of the liver and spleen of tumor-bearing mice, as compared to controls, is not an indirect effect due to a change in total phosphorus metabolism—e.g., an increase in cell permeability to phosphorus, but is probably due to an increase in cell proliferation. To substantiate this observation further, formate-C^{14} and glycine-2-C^{14} were employed as precursors in a similar experiment.

EXPERIMENTAL

Male A strain mice were used in all the experiments. The mice were fasted for 24 hours before being sacrificed. The nucleic acid turnover was measured by giving a tracer dose intraperitoneally of formate-C^{14} or glycine-2-C^{14}, sacrificing the mice after 4 hours, isolating the DNA from livers, intestines, spleens, and tumors, and the cPNA from livers, and measuring the specific activity of the nucleic acid. The specific activity is expressed as the percentage of incorporation of the injected dose per milligram of nucleic acid phosphorus.

The method used for the isolation of the DNA from spleen, intestine, and tumor was essentially Levene's, except for some changes which had been found necessary to make it suitable for a tracer experiment involving C^{14} (2, 5). The isolation of the DNA and cPNA from the livers has been described in two earlier papers (5, 6). The DNA was extracted in a boiling mixture of sodium hydroxide plus sodium acetate. The protein was precipitated out with glacial acetic acid plus ferric hydroxide and centrifuged off. The nucleic acid was precipitated from the supernatant fluid with hydrochloric acid plus methyl alcohol. The precipitated nucleic acid was dissolved in 1 M sodium hydroxide plus an equal volume of methyl alcohol. Impurities insoluble in this solution were removed by centrifugation. The DNA was then reprecipitated 5 times with hydrochloric acid plus methyl alcohol, at which time a constant specific activity was obtained, which did not change upon further reprecipitation. This was taken as an indication that the nucleic acid was free from C^{14} contaminants. The isolation of the DNA and cPNA from the livers was described in two earlier papers (5, 6). It consisted primarily of separating the nuclei from the cytoplasm by differential centrifugation. The DNA was isolated from the nuclear fraction as described above. The cPNA was obtained from the cytoplasm by trichloroacetic acid (TCA) precipitation. The TCA-precipitated protein was suspended in ice-cold sodium hydroxide for 2 hours in order to split the protein-nucleic acid linkage. The protein was precipitated with glacial acetic acid and centrifuged off. The nucleic acid was precipitated out of the supernatant fluid with hydrochloric acid and methyl alcohol, and then reprecipitated 5 more times. At this point the specific activity remained constant and did not change upon further reprecipitation.

* Partially supported by the Atomic Energy Commission.

Received for publication January 21, 1952.

RESULTS AND DISCUSSION

As can be seen in Tables 1 and 2, the increase in specific activity observed earlier with the use of P^{32} in liver and spleen DNA was also observed with formate-C^{14} and glycine-2-C^{14}, while the cPNA specific activity in the tumor-bearing mice did not vary significantly from the control groups, except in the group of mice bearing sarcoma A274 transplants. The cPNA specific activity in this group was lower than the cPNA average for the entire group of control mice. However, the average cPNA value of the control mice which were used the same day as the sarcoma A274 mice was 4.6, which is not significantly different from 5.09, the average cPNA value obtained for the sarcoma A274 mice.

The specific activity of the DNA of the intestine was significantly lower in all the tumor-bearing mice, as compared to the controls.

LePage and Heidelberger (4) studied the incorporation of glycine-2-C^{14} into liver DNA and PNA and into tumor DNA, in normal rats and in rats bearing multiple Flexner-Jobling carcinoma. They determined the specific radioactivity at 12, 24, and 48 hours after the administration of the glycine by stomach tube. They observed the following: essentially no difference in the specific activity of liver DNA of control rats from that of rats bearing tumor transplants and also no significant difference in the specific activity of tumor DNA, liver DNA, and liver PNA. They interpreted their re-

TABLE 1

Specific Activity* × 10^4 of Nucleic Acid Labeled with Formate-C^{14}†

Control

Liver DNA‡	Liver cPNA‡	Spleen DNA§	Intestine DNA#
3.14	8.55	70.4	98.7
1.62	11.65	70.8	85.3
3.78	14.22	66.2	88.9
1.22	8.68	82.6	73.0
1.88	8.38	99.0	79.1
2.10	12.85	81.8	74.0
2.36	8.66	83.8	89.1
1.15	9.81	108.6	107.0
2.20	5.43		78.7
4.57	2.17		47.7
3.17	2.60		81.6
2.18	10.32		67.2
1.63	2.47		65.7
3.57			85.8
2.28			74.3
2.46±0.27 Av.	8.13±1.1	82.9±5.5	79.7±3.8

Mammary Carcinoma

Liver DNA‡	Liver cPNA‡	Spleen DNA§	Intestine DNA#
5.23	6.53	211.0	57.9
8.22	5.74	168.0	74.0
11.65	8.58	191.0	55.5
9.53	9.90	225.0	66.9
5.34	6.70	160.0	69.6
4.26	8.84	187.0	61.0
14.15	7.79	185.0	72.0
7.40	7.84		59.5
8.86	6.13		60.0
6.22	9.01		68.2
8.23	12.35		63.5
7.78	11.44		68.4
6.26	8.37		71.6
6.19	11.63		73.1
10.78	8.57		67.2
8.01±0.7 Av.	8.63±0.47	190±9	65.9±1.6

TABLE 1—*Continued*

Statistical Comparison of Mean Values for Specific Activity in Control Mice with Those in Mice Bearing Mammary Carcinoma

	Liver DNA	Liver cPNA	Spleen DNA	Intestine DNA
Probability of significance P	*0.001*	0.7	*0.001*	*0.01*

Sarcoma A274

Liver DNA‡	Liver cPNA‡	Spleen DNA§	Intestine DNA#	Tumor DNA‖
12.75	5.00	142.7	44.0	84.3
17.38	5.68	143.6	72.7	61.7
20.49	6.85	178.7	60.1	71.1
10.34	3.17		67.3	80.0
19.17	4.74		50.4	74.7
21.43			45.2	
16.92±2.2 Av.	5.09±0.68	155±14.0	56.6±5.6	74.4±4.4

Statistical Comparison of Mean Values for Specific Activity in Control Mice with Those in Mice Bearing Sarcoma A274

	Liver DNA	Liver cPNA	Spleen DNA	Intestine DNA
Probability of significance P	*0.001*	0.07	*0.03*	*0.015*

* Specific activity expressed as counts/minute/milligram of nucleic acid phosphorus divided by counts/minute injected, normalized for the weight of the mouse.

† Dose injected per mouse = 20.3 μc. of C^{14} = 1.06 mg. of formate in 0.1 cc. isotonic saline.

‡ Each value represents two livers.

§ Each value represents four spleens.

Each value represents two intestines.

‖ Each value represents tumor tissue from two mice.

Note: numbers in italics represent significant difference.

TABLE 2

Specific Activity* × 10^4 of Nucleic Acid Labeled with Glycine-$2C^{14}$*

Controls

Liver DNA†	Liver cPNA†	Spleen DNA‡	Intestine DNA§
2.18	6.20	19.0	33.6
1.87	9.43	17.2	17.1
1.96	8.61	16.6	31.5
1.63	6.49	16.0	21.3
3.43	6.54	15.8	17.9
2.58	8.23		22.5
1.73	8.62		13.7
1.62	6.07		18.5
1.44	8.12		16.7
1.98	8.53		16.4
5.63	7.45		18.6
2.37±0.38 Av.	7.66±0.37	16.9±.62	20.7±1.9

Mammary carcinoma

Liver DNA†	Liver cPNA†	Spleen DNA‡	Intestine DNA§	Tumor DNA#
5.20	15.3	30.5	11.6	11.3
4.64	7.8	33.6	15.0	13.2
6.48	6.7	36.4	11.9	12.5
3.58	7.7	41.3	11.7	12.1
3.93	10.9	45.3	12.4	23.4
4.27	9.1		11.8	11.1
5.17	9.2		15.6	11.6
3.30	10.0		16.9	10.9
4.46	9.1		12.8	11.7
4.09	8.0		15.5	
4.51±0.31	9.4±1.2	37.4±0.3	13.5±0.7	13.1±1.4

Comparison of Specific Activity of Control Mice with That of Mice Bearing Mammary Carcinoma‖

	Liver DNA	Liver cPNA	Spleen DNA	Intestine DNA
Probability of significance P	*0.01*	0.2	*0.001*	*0.01*

* Dose injected per mouse = 7.34 μc C^{14} = 0.57 mg. of glycine in 0.2 cc. isotonic saline.

† Each value represents two livers.

‡ Each value represents four spleens.

§ Each value represents two intestines.

Each value represents tumor tissue from two mice.

‖ Numbers in italics represent significant difference.

sults as indicating that there are two separate pathways of DNA synthesis (1).

We have observed an increase in liver DNA in mice and rats bearing tumor transplants compared to liver DNA from control mice and rats with every tumor thus far studied (2, 3, 5); however, this does not include the Flexner-Jobling carcinoma. In the present experiments we have obtained a ratio of tumor DNA:liver DNA of 5.5 employing glycine-2-C^{14} as the labeling agent, while we have observed a ratio of 70 using formate-C^{14} as the tracer. We have also observed this difference between the two precursors when the intestine DNA:liver DNA and spleen DNA:liver DNA ratios are compared. This may be explained by our preliminary finding that the liver takes up a proportionately larger amount of glycine than formate, whereas the amounts in spleen and intestine are approximately equal. This means that the availability of glycine for the formation of nucleic acid in the intestine and spleen is much less than the availability of formate in these tissues. The liver cPNA:DNA ratio is the same for both glycine and formate, namely, 3.1.

From the present experiments we can conclude that the increase in specific activity of liver and spleen DNA in tumor-bearing mice as compared to that in control mice with the use of radioactive phosphorus was due to a true change in the DNA turnover in these two tissues.

SUMMARY

The incorporation of formate-C^{14} and glycine-2-C^{14} into DNA of liver, spleen, intestine, and tumor tissue and into cPNA of liver was measured, 4 hours after the administration of the precursor, in male A strain mice bearing transplants of mammary carcinoma or sarcoma A274. An increase in the specific activity was observed in the liver and spleen DNA of tumor-bearing mice as compared to the DNA of the same tissues from control mice. The specific activity of the DNA of the intestine was lower in all the tumor-bearing mice than the controls. The liver cPNA specific activity was essentially the same in the mice with tumor transplants as in the control mice.

ACKNOWLEDGMENTS

The authors are very grateful to Mrs. Jean Siri for performing the tumor transplants. We are indebted to Dr. Bert M. Tolbert for supplying the formate-C^{14} and the glycine-2-C^{14}.

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V_2 Carcinoma in the Rabbit Eye*

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The relationship of the papilloma virus to a carcinoma derived from the virus-induced rabbit papilloma has been the subject of numerous studies. Early work indicated that the carcinoma (V_2) did not contain papilloma virus demonstrable by the usual infectivity tests. Nevertheless, there was reason to believe that papilloma antigen was present in the V_2 carcinoma cell, since the host rabbits developed specific papilloma complement-fixing antibodies during the course of intramuscular passage of the carcinoma (7). However, some time between the fifth and eighth years of continuous passage, a change occurred in the antigenic make-up of the V_2 carcinoma so that host rabbits no longer developed papilloma antibodies. Despite the apparent loss of papilloma antigen, the V_2 carcinoma has retained all its original characteristics (6). Recent studies of seven other papilloma-derived carcinomas have produced significant new findings (9). When these tumors were established in baby domestic rabbits, it was discovered that the carcinomas elicited little or no papilloma antibody; but small amounts of papilloma virus were demonstrated in three of six tumors. Later, when the same carcinomas were transferred to adult domestic rabbits, papilloma virus could no longer be demonstrated in extracts of the carcinomas, although the rabbits bearing the tumors did develop specific papilloma complement-fixing antibodies in their sera.

It has long been recognized that it was difficult, or often impossible, to demonstrate papilloma virus in the papillomas of domestic rabbits, although large amounts of virus usually could be isolated from cottontail rabbit papillomas (10). Therefore, it seemed probable that the papilloma virus, if present in V_2 carcinomas, would be difficult to demonstrate when the tumor was growing in the domestic rabbit. Therefore, from time to time, unsuccessful attempts have been made to establish the V_2 tumor in cottontail rabbits with the hope that the papilloma virus could thereby be isolated.

In the present studies of the V_2 carcinoma, it it was found that the tumor could be successfully transplanted to the anterior chamber of the domestic rabbit eye. By using V_2 carcinoma tissue derived from tumor growing in the domestic rabbit eye, it was also possible to transfer V_2 carcinoma into a series of cottontail rabbits in the form of iris tumors. This paper describes the characteristics of the V_2 eye tumor and the results of serologic and virus tests on the eye tissues and fluids.

MATERIALS AND METHODS

The V_2 carcinoma was obtained from Dr. John G. Kidd. This tumor has been continuously passed since 1938 by the intramuscular route in domestic rabbits. It has been maintained in this laboratory since 1948 by intramuscular inoculation of tumor cells at approximately monthly intervals. Adult New Zealand white male rabbits, purchased from a local dealer, were used in experiments with domestic rabbits. Cottontail rabbits were purchased from a Kansas trapper.

Intraocular inoculation.—V_2 tumor tissue was passed through a monel metal sieve (40 mesh) with 0.9 per cent saline and was centrifuged 5 minutes at 800 r.p.m. The sediment was diluted with an equal amount of saline containing penicillin and streptomycin in a final concentration of 200 units and 200 μg/ml, respectively. The suspension was then inoculated in 0.05-ml. amounts into the anterior eye chamber with a 22-gauge needle by a technic previously described (8).

Harvest of eye tumors and eye fluids.—The rabbits were killed by intravenous injection of air. The eyes were aseptically removed. Adherent blood and tissue were washed or cut away. The eye was then opened in a mortar. The iris and tumor were cut away; the lens was discarded. Aqueous and vitreous humors from both eyes of a given rabbit were pooled and separated from the tissue fragments. After clarification by centrifugation at 2,000 r.p.m. for 20 minutes, fluids were stored (−50° C.) undiluted or diluted tenfold in 0.9 per cent saline containing 200 units of penicillin and 200 μg of streptomycin/ml. Ten or 20 per cent extracts of V_2 iris tumors were made in 0.9 per cent saline. After light centrifugation, the materials were frozen at −50° C. until used in infectivity tests or until further prepared for complement fixation tests.

Sections for microscopic examination were made from tumor and adjacent tissue or the whole eye.

Complement fixation.—The procedure of Casals and Palacios was employed (1). Papilloma and V_2 carcinoma antigens were prepared as previously described (3, 5). All antigens were centrifuged at 2,000 r.p.m. for 30 minutes before use. Appropriate positive and negative control antigens and sera were included in all tests.

Tests for papilloma virus.—Eye fluids and extracts of eye

* This investigation was supported by a research grant from the National Cancer Institute of the National Institutes of Health, Public Health Service.

Received for publication February 8, 1952.

tumors were inoculated on the hyperplastic skin of domestic rabbits according to a previously described technic (2).

EXPERIMENTAL

V_2 tumors in the anterior eye chamber of the domestic rabbit.—V_2 carcinoma was obtained from a 3-week-old intramuscular growth in a New Zealand white rabbit. Necrotic material was removed, and healthy tumor tissue was passed through a sieve and injected into the anterior eye chambers of New Zealand white rabbits as described in "Methods."

Immediately after inoculation, the visible tumor fragments settled to the iris angle at the inferior border of the anterior eye chamber. A diffuse conjunctivitis developed within 24 hours and persisted for 3 days. By the fourth day, the injected tumor fragments were no longer visible. Evidence of tumor growth, as indicated by generalized iris edema and linear red and gray lines radiating to the pupil, was first noted the fifth day. From the seventh to fifteenth day, the entire iris rapidly became enlarged and thickened by increasing tumor infiltration. In the 4 days before harvest, progressive edema and bulging of the cornea were observed, and the color of the tumor appeared to change from gray to white. The eyes were harvested 15 days after inoculation, when one of the corneas burst. Gross examination of the eyes after removal of the cornea revealed that the abnormalities were confined to the iris, ciliary body, and the cornea (Fig. 1). Microscopic examination of whole eye sections revealed that the tumor cells observed in the iris and ciliary body appeared to be identical with the type of cell seen in V_2 intramuscular growths (Fig. 4).

A suspension of the V_2 tumor cells from the eyes of the first rabbit passage was prepared and injected into the anterior chamber of both eyes of two normal domestic rabbits. Tumor growth was similar to that of the first passage, and the eyes were harvested after 11 days. Thereafter, consecutive passages were made at 9–12-day intervals until a total of seven passages was made. Tumor growth was uniformly successful in both eyes of all rabbits. The size and extent of the tumor was similar in all eyes. Later passages did not grow faster than the initial passages. Histologic examination of eyes from later passages revealed that occasionally V_2 cells infiltrated eye structures adjacent to the ciliary body. Detailed studies to determine the minimal amount of tissue required to initiate growth were not carried out. However, suspensions containing 1 part of cells and 32 parts of diluent caused the development of tumors which were only slightly smaller than the usual ones. V_2 carcinoma from intramuscular growths was used to initiate three additional series of iris tumors. The original findings were duplicated in each series.

V_2 tumor in the anterior eye chamber of the cottontail rabbit.—The next experiments were designed to determine whether the V_2 carcinoma would grow in the anterior chamber of the cottontail rabbit. It was found that the tumor grew in the iris of both eyes of two cottontail rabbits inoculated with an aliquot of the same V_2 intramuscular tumor suspension that was employed to initiate the first eye passage in domestic rabbits. Growth of the tumor followed the same general pattern already described in detail for the domestic rabbit eye tumors. When the eyes were harvested 15 days after inoculation, it was noted that white tumor masses occupied the entire iris. Histologic examination revealed no significant differences from the microscopic appearance of the V_2 tumor in the domestic rabbit eye.

Further passages of the iris tumor were attempted. A suspension of the first passage eye tumors was inoculated into both eyes of two more cottontail rabbits. At harvest 12 days later, localized iris tumors 5×10 ml. in size were noted. A third passage with the use of the small tumors of the second passage produced only minute growths in the iris, which were insufficient for passage.

An attempt was then made to produce transplantable tumors by inoculating V_2 tumor tissue obtained from the domestic rabbit eye. Third passage V_2 eye carcinoma (domestic rabbit), harvested 12 days after inoculation, was prepared and inoculated in both eyes of two cottontail rabbits as described in "Methods."

In the first passage, white tumors with sharp borders developed and occupied one-half to two-thirds of the iris surface. Thereafter, six successful passages were carried out at 11- to 19-day intervals, with V_2 tumor from the preceding passage. Certain differences from the growth of V_2 eye tumor in the domestic rabbit were apparent. Although the tumors were confined to the iris, sharply localized single or multiple tumors were noted instead of complete infiltration of the iris. Only 75 per cent of the inoculated eyes developed tumors. The histologic character of the V_2 cells showed no definite differences from those of other V_2 tumors in the anterior eye chamber.

A year after the first experiments with cottontail rabbits, fresh domestic rabbit V_2 iris tumor cells were used to initiate further cottontail eye tumors. The resulting growths differed from those of the first series in that they were smaller and not always confined to the iris. Ap-

proximately one-third of the tumors extended across the pupil from the adjacent border of the iris.

Serologic and virus studies.—Before tests were made of eye fluids and tumors and of the sera from rabbits with V_2 eye tumors, complement fixation tests were performed with sera from domestic rabbits bearing V_2 intramuscular tumors. Tests of the sera from rabbits of fourteen consecutive passages revealed no complement-fixing antibodies for the papilloma virus. This confirmed the findings of Smith, Kidd, and Rous (11).

Eye fluids and eye tumor extracts from sixteen domestic rabbits representing seven eye passages were tested for evidence of papilloma complement-fixing antigen without success. In twelve of these rabbits, infectivity tests also failed to demonstrate papilloma virus in the eye fluids or tumors. Although papilloma complement-fixing antibodies could not be detected in any of sixteen domestic rabbit sera, four eye fluids were also tested for papilloma complement-fixing antibodies. These later tests were also negative. Therefore, there was no evidence of local antibody formation that might have masked papilloma virus.

Tests next were made with materials from cottontail rabbits bearing V_2 eye tumors despite the lack of evidence of papilloma antigen in domestic rabbit V_2 intramuscular and eye tumors. Again, eye fluids and eye tumors were tested for papilloma complement-fixing antigen. These tests were negative in four cottontail rabbits. No papilloma virus was demonstrated in infectivity tests employing whole eye extracts from ten additional cottontail rabbits bearing V_2 eye tumors. Finally, in the sera of the latter ten cottontails, no papilloma complement-fixing antibodies could be detected.

Previous studies (3) have shown that the blood of rabbits carrying the V_2 carcinoma contains antibodies which will fix complement in the presence of saline extracts of various normal and neoplastic rabbit tissues. The next experiments were performed to determine whether a distinctive antigen could be demonstrated in extracts of the V_2 eye tumors or in the fluids from these eyes. Extracts of eye tumors were prepared, and fluids were obtained from twenty eyes of domestic rabbits and ten eyes of cottontail rabbits with V_2 eye tumors.

Repeated tests failed to reveal a specific complement-fixing antigen in the eye fluids from rabbits with V_2 eye tumors. These results are in keeping with previous findings that the complement-fixing V_2 antigen is associated with a sedimentable particle contained in V_2 carcinoma. The results of tests with saline extracts of the eye tumors were similar to those previously obtained with extracts of the intramuscular growths (3).

DISCUSSION

The striking features of the growth of V_2 carcinoma in the anterior eye chamber of the domestic rabbit were the apparent disappearance of the tumor cell inoculum before tumor growth was noted in the iris and the diffuse multicentric origin and rapid growth of tumor over the entire iris surface. With the possible exception of the Kato sarcoma (4), this sequence of events has not been previously described. Usually, tumors have been implanted in the iris angle of the eye in the form of a single piece of tumor tissue. After a variable period of time, the tumor tissues have grown and have become attached to the iris or other eye structures. It is likely that the diffuse type of iris tumor growth seen with the V_2 tumor is attributable to the physical character of the inoculum rather than to any special character of the V_2 carcinoma. In the act of inoculation, minute tumor fragments may diffusely seed the iris. The tumor inoculum that disappeared from its settling place was probably completely absorbed and had nothing to do with the growth of V_2 carcinoma in the iris. It seems certain that no soluble tumor substance invades the iris, because inoculations of cell-free fluids from eyes with the V_2 tumor failed to produce eye tumors. Furthermore, all previous attempts to pass V_2 carcinoma with cell-free extracts of intramuscular growths have been unsuccessful.

One of the primary objects of this study was to attempt to demonstrate papilloma virus or antigen in the V_2 carcinoma by growing the tumor in the cottontail rabbit. Although it was possible to grow and to transfer serially the V_2 carcinoma in the cottontail anterior eye chamber, attempts to demonstrate papilloma virus or antigen in cottontail V_2 tissue were unsuccessful. As indicated, however, there is evidence that the papilloma antigen had been lost from the V_2 carcinoma before its passage to the cottontail eye. Successful transfer of the more recently established papilloma-derived carcinomas to the cottontail eye might make it possible to demonstrate larger amounts of papilloma virus in the tumors (9).

SUMMARY

1. V_2 carcinoma was successfully grown and carried through seven consecutive passages in the anterior eye chamber of the domestic rabbit. Rapid confluent growth in the iris and ciliary body necessitated the harvest 9–12 days after inoculation to prevent rupture of the cornea.

2. V_2 carcinoma from the eyes of domestic rab-

bits was utilized to initiate six consecutive passages of V_2 tumor in the anterior eye chamber of the cottontail rabbit. Rapid growth occurred as single or multiple isolated tumors on the iris and ciliary body in 75 per cent of the inoculated eyes.

3. Confirmatory evidence was obtained that the V_2 carcinoma no longer carries a masked papilloma antigen. No papilloma virus nor any papilloma complement-fixing antigen was detected in the eye fluids or the eye tissues of domestic or cottontail rabbits with V_2 eye tumors. Complement-fixing antibodies against the papilloma virus were not demonstrable in the sera of rabbits with V_2 eye or V_2 intramuscular tumors.

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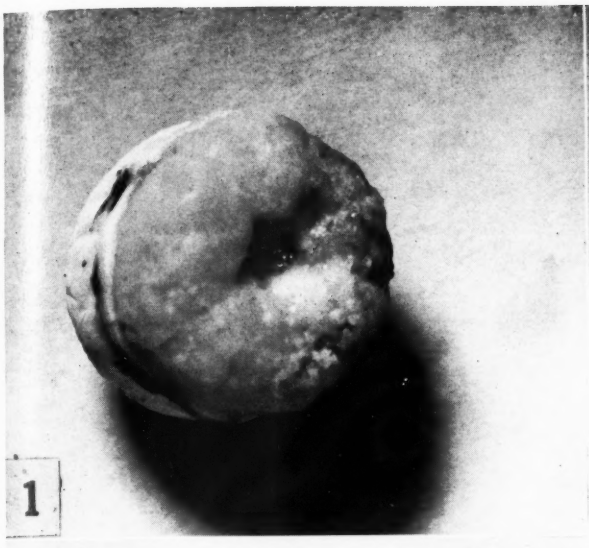
Fig. 1.—V_2 carcinoma growing in the iris and ciliary body of domestic rabbit eye. The cornea has been removed. (2 × actual size.)

Fig. 2.—Normal domestic rabbit eye for comparison with Figure 1. The cornea has been removed. (2 × actual size.)

Fig. 3.—V_2 carcinoma in the iris and ciliary body of domestic rabbit eye. Hematoxylin and eosin stain. ×7.

Fig. 4.—Detail of V_2 carcinoma cells in Figure 3. ×450.



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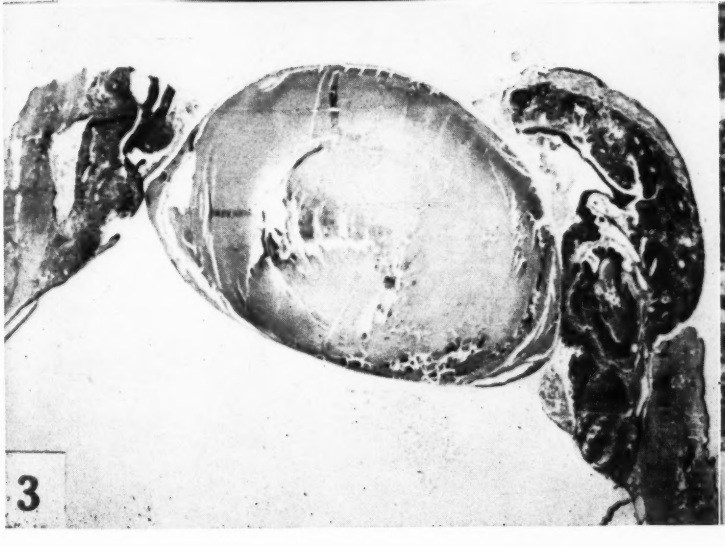

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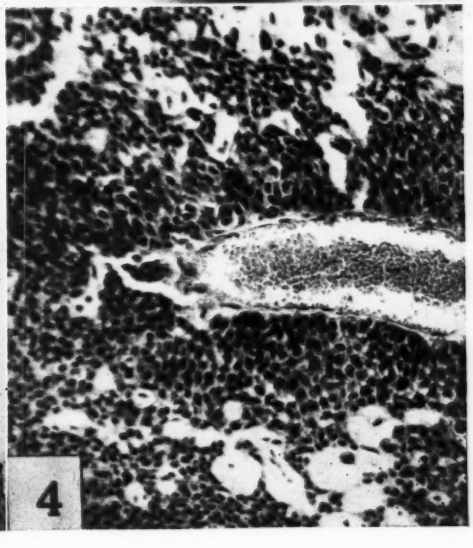

4

The Effect of Radiation on Transplantable Mouse Tumor Cells

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In previous publications (2, 3) it was shown that the growth rate of a transplantable mouse adenocarcinoma was exponential with time within the limits of the growth period imposed by the experiment and that the percentage of animals which developed tumors depended on the number of viable cells inoculated. For this paper the same technic was used to study the effect of x-radiation on tumor cells in suspension: first, to establish the lethal effect of equal doses of radiation on different concentrations of tumor cells; second, to determine the influence of radiation on the growth period; third, to determine whether any radiation effect would be transmitted to a second transplant generation; and, fourth, to determine the lethal effect of other doses of radiation delivered to the tumor cell suspensions.

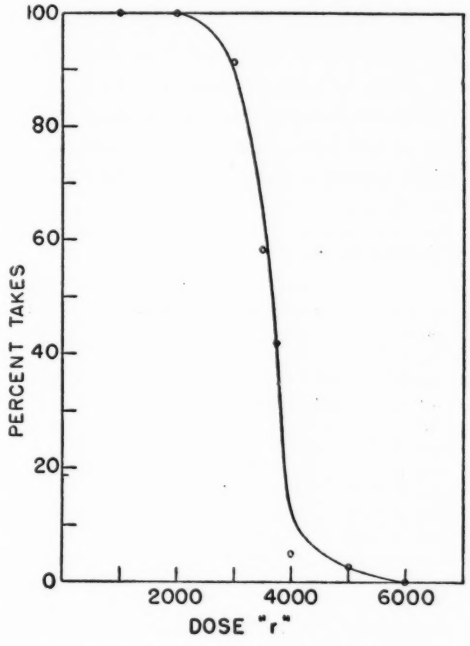

Chart 1.—Survival curve of the Marsh-Simpson tumor irradiated *in vivo* and transplated immediately.

METHODS

The tumor selected for this work was the same as that used in the previous work, namely, the Marsh-Simpson tumor which has been propagated by successive transplantations in the Marsh strain of albino mice. The percentage of takes of this tumor in this strain is consistently 100 per cent.

The radiation was generated at 200 kv. with a filter of 0.5 mm. copper, which produces a beam having a HVL of 0.9 mm Cu. The dose rate was 120 r per minute at 30 cm. distance.

From previously unpublished data, the lethal effect of varying doses of *in vivo* radiation on this tumor was determined by exposing tumor-bearing mice to a beam of x-radiation of the same quality as described above, excising and transplanting the tumors immediately following exposure to the rays. The data from these determinations are plotted in Chart 1. From this curve it can be determined that the 50 per cent lethal value is approximately 3,600 r.

For the preliminary tumor cell suspension exposures, doses of 3,600 r were used, the 50 per cent lethal dose for tumors irradiated *in vivo*. When the cell suspensions were subjected to this dose, however, no growth occurred in the inoculated animals. Subsequent exposures made at lower doses showed that 500–1,000 r permitted growth in a sufficient number of animals so that both the percentage of takes and the growth period could be determined. The data on these suspensions which were irradiated with 500 r and inoculated immediately following the radiation into test animals are assembled in Table 1. The data include the viable cell count made immediately before radiation, the number of animals used for each suspension, and the percentage of takes. A plot of these data shows the relationship between the concentration of the viable cells and the percentage of takes (Chart 2). It should be noted that each of suspensions 124–150 was divided into two portions after the

TABLE 1

Results of the Inoculation of Suspensions Irradiated with 500 r

Experiment no.	No. cells inoculated $\times 10^3$	No. mice	No. takes	Percentage takes	
126	0.333	63	0	0.0	
124	0.677	41	1	2.4	
128	2.66	52	3	5.8	
143	3.25	50	10	20.0	
144	4.16	45	23	51.0	
152	4.83	39	20	51.2	
138	4.9	40	19	47.5	
150	4.94	31	11	35.5	
149	5.88	38	17	44.7	
136	6.0	55	39	71.0	
133	6.25	38	31	81.5	
155	12.9	48	46	96.0	
131	19.25	51	42	82.2	
142	134.0	40	40	100.0	
223	5.15	37	17	46.0	(Final average volume not determined because these tumors were used for reinoculation. See Table 5.)
221	5.62	41	33	80.5	
212	7.20	19	15	79.0	
217	10.95	23	21	91.3	
205	115.00	30	30	100.0	

Received for publication February 10, 1952.

determination of viable cell count; one was irradiated and inoculated immediately, while the unaltered portion was inoculated simultaneously into another group of mice of the same age, so that there was a control group of mice for each suspension. No controls were made for suspensions 212–223, because the tumors arising from these irradiated suspensions were to be used for another experiment.

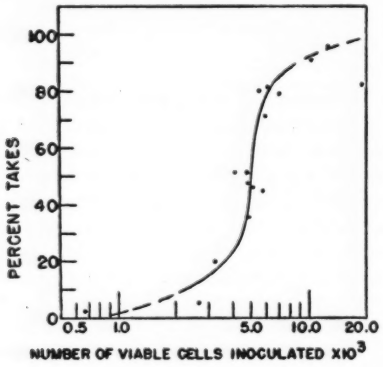

CHART 2.—Relation between number of viable cells irradiated *in vitro* with 500 r and the percentage of animals in which the tumor grew.

published curve on the percentage of takes (Chart 3). It can be observed that the more recent values —that is, the values for the controls for the irradiated suspensions—fall very well along the curve, except for the higher concentrations, where there is a slight departure in the direction of higher concentrations. This portion of the curve has been modified in view of these additional data, as shown by the dotted portion of the curve.

A comparison of the numbers of viable cells required to produce the same percentage takes for the control suspensions (Table 2) and the 500-r irradiated suspensions (Table 1) is given in Table 3. In this table it can be noted that the concentrations of viable cells in the irradiated suspensions necessary to produce the same percentage of takes as in the controls is markedly greater, for all suspensions. This is interpreted as indicating that the difference between the two is a measure of the killing effect of the radiation (see column 4, Table 3). This killing effect is not constant for all concentra-

TABLE 2

CONTROLS FOR THE IRRADIATED SUSPENSIONS

Exp. no.	No. cells inoc. $\times 10^3$	Vol. cells inoc. (cc.)	No. mice	No. takes	Per cent takes	Av. growth (days)	Final av. vol. (cc.)	Growth constant (μ/day)
126	0.333		52	5	9.7			
124	0.677		30	5	16.8			
128	2.66	1.45×10^{-6}	48	32	74.4	33.9	0.097	0.327
143	3.25	1.79×10^{-6}	30	25	83.2	36.5	0.103	0.300
181	3.81	2.1×10^{-6}	19	17	89.5	29.1	0.096	0.370
144	4.16	2.3×10^{-6}	31	30	97.0	33.6	0.092	0.314
152	4.83	2.66×10^{-6}	17	17	100.0	35.5	0.104	0.299
138	4.9	2.7×10^{-6}	19	19	100.0	31.5	0.111	0.336
150	4.94	2.72×10^{-6}	22	22	100.0	31.4	0.106	0.340
149	5.88	3.24×10^{-6}	32	32	100.0	32.8	0.115	0.320
136	6.00	3.31×10^{-6}	31	31	100.0	29.4	0.105	0.352
177	6.09	3.36×10^{-6}	20	19	95.0	32.7	0.108	0.316
133	6.25	3.4×10^{-6}	37	37	100.0	25.8	0.084	0.387
155	12.90	7.12×10^{-6}	19	19	100.0	24.4	0.193	0.420
178	13.40	7.39×10^{-6}	23	23	100.0	21.9	0.139	0.448
170	16.9	9.3×10^{-6}	24	24	100.0	23.8	0.128	0.401
131	19.25	1.06×10^{-5}	49	49	100.0	26.0	0.085	0.364
173	31.0	1.71×10^{-5}	21	21	100.0	24.3	0.163	0.378
162	57.9	3.19×10^{-5}	19	19	100.0	24.3	0.148	0.342
188	112.0	6.18×10^{-5}	18	18	100.0	21.0	0.155	0.374
142	134.0	7.37×10^{-5}	13	13	100.0	18.7	0.110	0.393

Both controls and animals which had received irradiated suspensions were examined semi-weekly, and, when the tumors were approximately 0.1 cc. in size, they were removed and the tumor volume measured. Those animals in which the tumor failed to grow were permitted to remain alive several weeks after the last tumor in that group had been measured—usually about 60 days.

RESULTS

The data for the control suspensions, including the controls for the 500-r, 1,000-r and 2,000-r irradiated suspensions, are given in Table 2. The experimental points for these suspensions, showing the relationship of the percentage of takes to cell concentration, are superimposed on the previously

tions of tumor cells but decreases with increasing concentrations of cells. The reverse or survival of cells is shown in column 5, Table 3. A plot of the percentage of cell survival against the percentage of takes is shown in Chart 4. The concentrations for the various percentages of takes are included in this graph. Furth *et al.* (1) have shown a comparable effect of radiation on leukemia cells.

The total growth period required for the irradiated suspensions to attain essentially the same final volume as their corresponding control suspensions is appreciably greater, as is shown in the data of Tables 2 and 4. But, since the curve on the percentage of takes shows that a definite number of

cells are destroyed by 500 r of radiation, depending on the original concentration of the suspension, it is necessary to take this fact into consideration and to reduce the original concentration of each irradiated suspension according to the curve in Chart 4. This would mean that each irradiated suspension actually started with a smaller volume of viable cells than its corresponding control and would of course require a longer growth period to attain the same final volume. However, even after these corrections were made for each suspension, it was evident that the growth period for the irradiated suspensions was longer than would be expected for a suspension of normal cells of that concentration (Table 4).

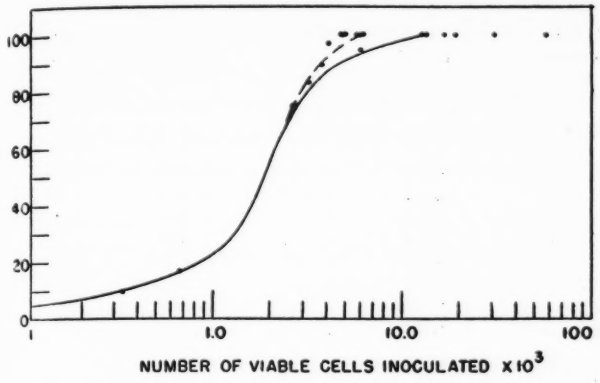

CHART 3.—Relation between number of viable cells inoculated and the percentage of animals in which the tumor grew.

TABLE 3

A COMPARISON OF THE NUMBER OF VIABLE CELLS REQUIRED TO PRODUCE THE SAME PERCENTAGE OF TAKES

Takes (per cent)	No. cells control $\times 10^3$	No. cells 500 r $\times 10^3$	Destruction (per cent)	Survival (per cent)
5	0.11	1.5	92.7	7.3
10	0.333	2.25	86.7	13.3
20	0.85	3.62	76.5	23.5
30	1.31	4.55	71.2	28.8
40	1.62	4.9	67.0	33.0
50	1.89	5.0	62.2	37.8
60	2.16	5.2	58.5	41.5
70	2.5	5.5	54.5	45.5
80	2.95	6.3	53.2	46.8
90	3.8	8.9	57.4	42.6

Two possible explanations for these findings may be made: (*a*) Those cells which survived the radiation grew at a slower rate. (*b*) Those cells which survived the radiation grew at the normal rate but were dormant for a period of days after the irradiation before resuming growth.

These two possibilities were examined for each suspension. The procedure followed may best be explained graphically, using suspension No. 133 as a typical example (see Chart 5). Point *A* represents the initial volume of viable cells for the control suspension. Point *B* represents the final average volume actually attained for the control suspension in 25.8 days. Line *AB* has a growth rate per day, μ, of 0.387. Point *D* indicates the final average volume of 0.068 cc. attained in 39.4 days for the irradiated suspension. Since the original volume *A* was reduced to 45.5 per cent by 500 r x-radiation (see Chart 4), the volume attained after 39.4 days of growth following irradiation must have started with a volume of 1.58×10^{-6} cc. This volume has been plotted as point *C* on the curve. By connecting points *C* and *D*, a growth constant of 0.303 per day is found for this suspension. Repeating this procedure for each of the irradiated suspensions, we find that the postirradiation growth constants are fairly consistent, as shown in Table 4.

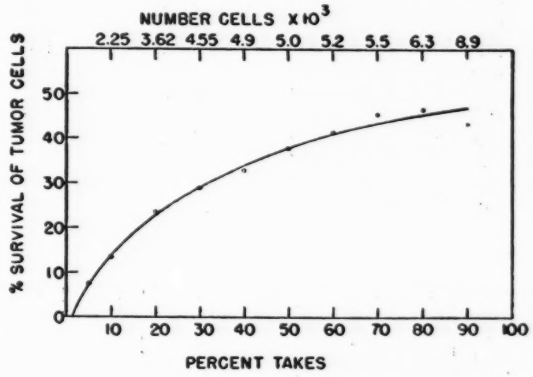

CHART 4.—Per cent survival of viable tumor cells as a function of the concentration when irradiated with 500 r.

On the other hand, if, after irradiation, the cells of this suspension had grown at a normal rate to produce the final average volume of 0.068 cc. in 39.4 days, then the growth rate would be represented by a line formed by projecting point *D* parallel to line *AB*. Such a line would have the same μ or growth rate per day as the control suspension, but would intercept the corrected volume at Point *E*, or 7.5 days after inoculation. This may be interpreted as indicating that these irradiated cells were in a dormant state for a period of 7.5 days, after which interval they proceeded to grow at a normal rate. Table 4, column 9, shows the dormant periods determined in this way for all the irradiated suspensions, the average being 7.2 days.

From the preceding discussion and calculations there is no direct proof as to which of the two procedures is taking place. If the growth rate is characteristic for each type of cell and a change in this rate had been produced by the x-rays delivered, then it might be a permanent change which could be measured in succeeding transplant generations.

If, on the other hand, the second possibility is true, namely, that the radiation caused the tumor cells to lie dormant for a short period after which normal growth occurred, then it would be reasonable to expect a normal growth rate when these tumors which grew after irradiation were transplanted into other mice. Therefore, the following experiments were made to determine whether any effects of radiation were transmitted to a second transplant generation.

A suspension was made of normal tumor cells, the concentration of viable cells determined and

TABLE 4

CALCULATED POSTIRRADIATION GROWTH CONSTANTS

Exp. no.	Vol. cells inoc. (cc.)	No. cells inoc. $\times 10^3$	Survival (per cent)	Corrected volume inoc.	Growth period (days)	Final vol. (cc.)	Growth constant	Dormant period (days)
126	1.83×10^{-7}	0.333						
124	3.74×10^{-7}	0.677	None or too few takes					
128	1.45×10^{-6}	2.66						
143	1.79×10^{-6}	3.25	20.4	3.65×10^{-7}	48.1	0.096	0.261	6.5
144	2.3×10^{-6}	4.16	26.5	6.1×10^{-7}	45.6	0.083	0.263	7.9
152	2.66×10^{-6}	4.83	32.2	8.56×10^{-7}	46.5	0.108	0.254	7.2
138	2.70×10^{-6}	4.90	34.0	9.18×10^{-7}	42.7	0.116	0.275	8.1
150	2.72×10^{-6}	4.94	35.2	9.57×10^{-7}	44.3	0.097	0.260	10.2
149	3.24×10^{-6}	5.88	44.6	1.45×10^{-6}	39.4	0.107	0.285	4.2
136	3.31×10^{-6}	6.00	44.8	1.48×10^{-6}	36.9	0.080	0.296	5.9
133	3.45×10^{-6}	6.25	45.5	1.55×10^{-5}	34.9	0.068	0.303	7.5
155	7.12×10^{-6}	12.9						
131	1.06×10^{-5}	19.25	Original concentrations beyond curve of Chart 4.					
142	7.37×10^{-5}	134.00						

Av. 7.2

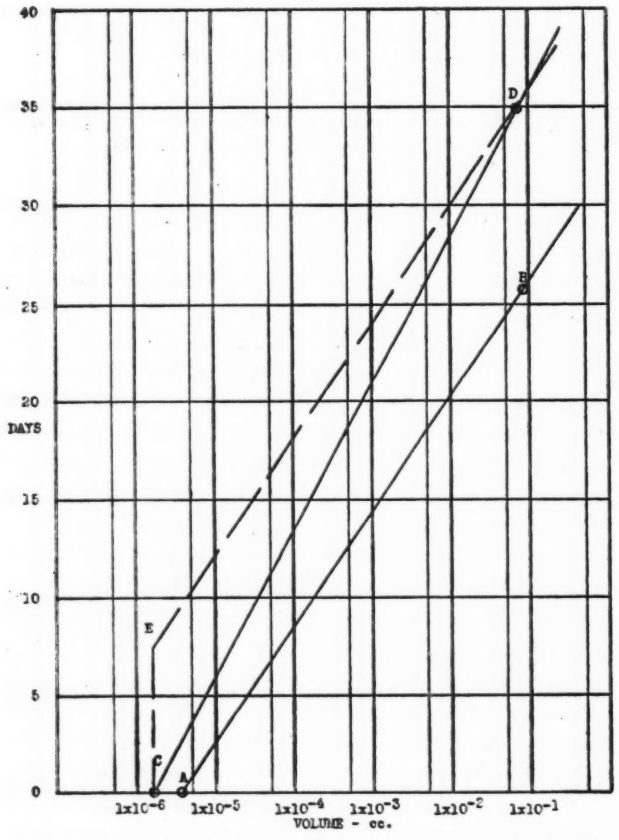

CHART 5.—Growth curves of tumors from suspension No. 133.

A–B Control suspension.
C–D Radiated suspension.
E–D Radiated suspension with same growth rate as control.
C–E Dormant period.

adjusted so that the tumor would grow in less than 50 per cent of the animals following irradiation. This suspension was irradiated with a dose of 500 r immediately before inoculation. Since considerable tumor material is required to make a suspension, these tumors arising from irradiated suspensions were permitted to grow to a size suitable for the preparation of an inoculum, and no endeavor was made to determine their final volume. These data are included for percentage of takes only, in Table 1. When relatively large tumors could be noted in a sufficient number of animals, suspensions were made, counted, and inoculated into a second group of mice. The data for the percentage of takes and growth constants for the second inoculation are shown in Table 5.

It is interesting to note that the growth constant and the percentage of takes agree with those of the controls and not with those of the irradiated suspensions. In other words, there is no evidence that the effect of radiation was carried over into the second transplant generation. As a matter of speculation, it can be observed that both the growth constants and the percentage of takes are slightly greater than the control values. This is indicative not only of complete recovery from the effects of radiation, but is also suggestive of an enhanced compensatory response.

Several suspensions were irradiated with a dose of 1,000 r and 2,000 r, the controls for which are included in Table 2. However, because of the very extensive destructive effect of these doses, it was

necessary to start with very high concentrations of cells. Such high concentrations are not only difficult to produce, but it is also difficult to obtain an accurate viable cell count. Because of these difficulties, too few suspensions were irradiated with these doses to permit a satisfactory estimate of the destructive effect and growth period. Such data as are available indicate that the destructive effect of 1,000 r varies between 97 per cent for the dilute suspensions and 80 per cent for the concentrated suspensions. The total growth period for the mice inoculated with suspensions irradiated with 1,000 r is considerably greater than that for mice inoculated with suspensions irradiated with 500 r; but, when the initial volume is corrected for the destructive effect, the growth rate is approximately the same as that for the 500-r suspensions. This would seem to be incompatible with the findings for the 500-r suspensions. If the cells surviving the 500-r dose grow at a reduced rate, it seems reasonable to assume that a dose of 1,000 r would still further depress the growth rate. On the other hand, if one assumes that the effect of radiation is to stun those cells which survive and to produce a condition of dormancy, then the data for the 1,000-r experiments could be interpreted as being indicative of a longer dormant period, which was found to be 15 days, or about double that for mice irradiated with 500 r. It must be kept in mind that the conclusions for the 1,000-r irradiated mice are based on only six suspensions and 274 mice.

With doses of 2,000 r there were too few takes to permit any estimates of growth period or destructive effect. However, the indications are a destruction of more than 99 per cent of the viable cells.

TABLE 5

RESULTS OF SECOND INOCULATION OF TUMORS ARISING FROM SUSPENSIONS IRRADIATED WITH 500 r

Exp. no.	No. cells inoc. $\times 10^3$	Vol. cells inoc. (cc.)	Mice inoc.	Per cent takes	Av. growth (days)	Av. final volume (cc.)	Growth constant (μ)
222	1.44	7.95×10^{-7}	35	65.7	27.3	0.222	0.458
225	2.28	1.26×10^{-6}	42	45.3	29.6	0.249	0.415
215	2.81	1.55×10^{-6}	39	94.8	25.4	0.111	0.438
219	2.97	1.64×10^{-6}	36	47.0	27.6	0.13	0.404
207	3.05	1.68×10^{-6}	39	95.0	27.4	0.103	0.399
210	5.94	3.28×10^{-6}	57	91.2	26.2	0.09	0.389

SUMMARY

1. The survival curve of a transplantable mouse adenocarcinoma is given when irradiated *in vivo* and transplanted immediately.

2. The destructive effect of a dose of 500 r on viable tumor cells in suspension was obtained by comparing the number of viable cells in an irradiated suspension with those in an untreated suspension producing the same percentage of takes.

3. The destructive effect of a 500-r dose of radiation increases with decreasing concentration of viable cells.

4. The radiation acts on those cells which survive, either to produce a lower growth rate or a period of dormancy of several days followed by a normal growth rate. The fact that tumors arising from an irradiated suspension fail to show any carry-over of the effects of radiation on either the percentage of takes or the growth rate, into the second transplantation, lead the authors to favor the latter suggestion.

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Studies on a Transplantable Teratoma of the Mouse*

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Ovarian teratomas are rare tumors in mice, and few cases have been mentioned in the literature. Transplantation studies with only one embryoma were reported by Jackson and Brues (3). This embryoma occurred spontaneously in the ovary of a C3H mouse and was successfully transplanted into mice of the same strain through eleven serial transplants. The pleomorphic nature of the embryoma was maintained *in vivo* and in tissue culture.

More recently, Fawcett (2) described bilateral ovarian teratomas found in a Swiss albino mouse and remarked about the infrequency of this type of tumor.

We are presenting observations on an ovarian teratoma that occurred in our mouse colony and has been maintained by serial transplantation. The tumor occurred in a mouse of the $C3H_eB$ substrain. This line was developed by transferring ova of a mouse of the C3H inbred strain, 2 days after fertilization, into the uterus of a C57BL/6 female. The descendants of the young born to the C57BL/6 mother, and nursed by her, were continued to be inbred by brother-sister matings, and constitute the $C3H_eB$ substrain.

The animal had a palpable abdominal tumor and was killed at the age of 216 days. The left ovary was greatly enlarged and measured approximately $10 \times 15 \times 20$ mm. The right ovary was normal. Parts of the enlarged left ovary were fixed and prepared for sectioning, and parts of it were used for transplantation. Microscopic examination showed that the ovarian tumor was composed of undifferentiated "embryonal" cells and many types of differentiated tissue. Remains of the ovary were present and contained a few atretic follicles and at least one normal follicle with ovum. Some of the undifferentiated cells were round or oval-shaped and were arranged in small nests (Fig. 1); others showed more varied forms and were scattered irregularly. Both types revealed mitotic figures. The differentiated tissues included nervous tissue (Fig. 2), hyaline cartilage, bone, striated muscle fibers (Fig. 3), and the following kinds of epithelia: stratified squamous, cuboidal ciliated, simple columnar, and goblet cells. These epithelial cells formed the lining of cysts of various sizes. Small groups of alveoli formed by cuboidal epithelium and occasionally surrounded by smooth muscle fibers were seen. At some areas abundant amounts of pigment granules were present in epithelial cells and in stellate cells. Mitotic figures were rare. The various tissues intermingled without evidence of organization. The tumor was designated as E 6496 and was diagnosed a teratoma.

For transplantation, several pieces of the original tumor were selected, cut into very small particles, and well mixed. The teratoma was transplanted subcutaneously with a trocar into five weaning age hybrids of C57BL/6 ♀ × C3H ♂. In about 4 weeks all five mice had palpable tumors at the site of transplantation. One of these animals was killed, and the tumor was retransplanted. Thereafter, subcutaneous transplantations were made about every 40 days. Samples from each tumor that was used for transplantation were sectioned for microscopic studies. At each successive transplantation care was taken to include areas of the tumor that differed in color and density. Therefore, several parts were cut up and minced well. An attempt was also made to separate the different components of the tumor: for example, small pieces of black pigmented parts, soft white parts, or hard bony parts, etc., were transplanted into identified mice. Many of the tumors resulting from these transplants were also sectioned.

To date, the tumor is in its ninth transplant generation. It grows in almost all the males and females of the $C3H_eB$ strain in which it originated, in the C3H strain, and in F_1 hybrids from C57BL/6 × C3H. Occasionally the tumor invades the abdominal wall, but so far metastasis has not occurred. The sizes attained by the tumors vary greatly. Three of the largest dissected tumors weighed 34, 30, and 29 gm. about 70 days after transplantation. The tumors kill the hosts in an average of 80 days.

* This investigation was aided in part by the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council and in part by the National Cancer Institute of the National Institutes of Health, Public Health Service.

Received for publication February 18, 1952.

At the second and third transfer generations, the tumor was transplanted subcutaneously into mice of the following unrelated strains: BALB/c, A/He, RIII/JFe, AKR, DBA/1 and C57BL/6. These animals were kept under observation for 5 months. In one of the RIII/JFe animals, a hard nodule of pin-head size was palpable at the site of the transplantation. Sectioned and examined microscopically, this proved to be a small piece of bone, which was probably present in the transplanted tumor and persisted in the new host. In another RIII/JFe mouse, a small area containing pigment granules was found at the site of the transplant. None of the animals showed any tumor growth.

At the seventh and eighth serial transfers, subcutaneous transplantations were done into the following foreign strains: C57L, DBA/1, C57BL/6, RIII/JFe, AKR, A/He and BALB/c. These animals have been under observation for 2–3 months and are still alive. None of them has any palpable tumors. Table 1 summarizes the results of transplantations in the related and unrelated strains.

Microscopic examination of sections of about 90 tumors showed that the tumors resulting from the transplantations, like the original teratoma, were mixed, containing undifferentiated "embryonal" and a variety of differentiated tissues. In general, the proportion of differentiated adult tissues was greater in large tumors which grew in the hosts for a longer period, than in the small tumors which were allowed to grow for a shorter time. The attempt to isolate different components of the tumor by transplanting small pieces of black pigmented, or soft white parts, etc., was not successful. In some cases these components were somewhat more conspicuous in the resulting tumors, but in all cases many other tissues were also present, and uniform growth of the isolated components did not result.

Figure 4 shows parts of the tumor in the first transplant generation. Nervous tissue, stratified squamous epithelium, striated muscle fibers, bone surrounded by fibrous connective tissue, and a cyst lined by ciliated columnar epithelium represent the differentiated tissues. The undifferentiated cells are irregularly scattered and show many mitotic figures. Figure 5 shows parts of a tumor of the second transfer generation in which cartilage, bone with marrow spaces, stratified squamous epithelium, and small alveoli are visible.

The fifth transplant generation is represented by Figures 6, 7, and 8. In Figure 6 a small cyst lined by stratified squamous epithelium and a large cyst lined partly by pigmented epithelium and partly by goblet and columnar cells can be seen. The goblet cells were functioning, and the cyst contained mucus, the product of their secretion. Figure 7 shows a few striated muscle fibers and loose connective tissue. In Figure 8 a tube is present, lined in parts by pigmented columnar and stratified squamous epithelium and by goblet cells intermingled with columnar epithelium. Finger-like structures resembling intestinal villi project into the tube. Transplantation of parts of the fifth generation tumor shown in Figure 6 resulted in the sixth generation tumor shown in Figure 9. The animal bearing this tumor was killed 82 days after transplantation. Cartilage, bone, dense fibrous connective tissue, small cysts lined by various types of epithelia, and nervous tissue can be seen. This tumor shows more differentiated tissues than the one in Figure 6 which grew in its host for only 48 days.

TABLE 1

RESULTS OF TRANSPLANTATION OF E 6496 OVARIAN TERATOMA INTO RELATED AND UNRELATED STRAINS OF MICE

RELATED STRAINS*		UNRELATED STRAINS	
Transplant generation	No.†	Host strain	No.†
First	5/5		
Second	14/14	BALB/c	0/4
		A/He	0/3
		C57BL/6	0/4
Third	24/24	AKR	0/3
		RIII/JFe	0/3
Fourth	29/31		
Fifth	29/29		
Sixth	27/27		
Seventh	15/15	C57BL/6	0/4
		C57L	0/4
Eighth	7/7	DBA/1	0/4
Ninth	6/6		

* The related strains include C3H$_e$B, C3H, and F_1 hybrids from C57BL/6 ♀ × C3H ♂.

† Numerators are the numbers growing the tumors; numbers in the denominators are the total number of animals receiving tumor transplants.

In all the sections examined, regardless of the degree of differentiation, the distribution of the various tissues was confused, and organization was almost completely absent.

It was evident in all the tumors that the differentiated adult tissues contained few mitotic figures, while the undifferentiated embryonal cells were proliferating rapidly. Mitotic figures in these cells were carefully studied to ascertain whether they contained the haploid or the diploid number of chromosomes. Chromosome counts of metaphase plates showed the chromosomes clearly to be diploid. On the basis of a quantitative study of mitotic figures, the growth rates of the original ovarian teratoma and tumors of the first and fourth transplant generations were compared.

Each of the transplanted tumors had been growing in its host for 41 days. The sections selected from all three of these tumors were approximately equal in size and contained about the same amounts of undifferentiated embryonal and differentiated adult tissues. The counts were made at a magnification of × 900 (oil immersion), and all the mitotic figures present in the sections were counted. There were 56 mitotic figures found in the section of the original ovarian teratoma, 477 in the section of the first transplant generation, and 369 in the section of the fourth transplant generation. The rates of growth in the first and fourth transplant generations were not significantly different from one another but were greatly increased compared to the original tumor. Perhaps it can be surmised that in the ovary the growth rate of the tumor was held in check in some way, and after transplantation into new hosts grew without such restriction.

DISCUSSION

There are many hypotheses regarding the origin of teratomas. According to Ewing, the origin of the ovarian teratoma is now referred by the great majority of observers to the sex cell or ovum. As was mentioned previously, chromosome counts of metaphase plates of teratoma E 6496 showed them to be diploid. Although this does not enable us to determine the origin, it rules out the possibility of haploid parthenogenetic development of this tumor from an ovum or polar body.

It is generally accepted that the undifferentiated embryonic tissues of teratomas are pluripotent and are able to give rise to cells that differentiate into the diverse components of these complex tumors. The fact that teratoma E 6496, transplanted into new hosts through nine serial transplants, maintained its pleomorphic structure is a proof of this pluripotency. Indeed, it is highly improbable that the amount of tissue introduced subcutaneously at each successive transplantation could contain all the diverse components found in the tumors of the new hosts. The varied elements could be produced only by the continuous growth and differentiation of transferred pluripotent cells. Attempts to separate and grow the differentiated components failed because complete separations were not accomplished, and, while the differentiated components may have persisted, actual growth depended on the undifferentiated pluripotent cells.

Studies of all the tumors show that the undifferentiated cells are growing most rapidly. These, therefore, are the most essential elements. In giving rise to the various tissues the undifferentiated cells show their potentialities and give proof of their pluripotency.

It is generally considered that teratomas containing embryonic components are usually malignant. Teratoma E 6496 contains such components.

The results with the transplantation of this tumor were similar to those of Jackson and Brues, who also observed that their embryoma maintained its pleomorphic nature through eleven serial transplants.

Subcutaneous transplantations of the teratoma into foreign strains did not result in progressive growth. In its specificity to grow only in the strain in which it originated, and in the F_1 hybrids of this strain, the teratoma behaved like most other mouse tumors.

The tumor will be maintained by transplantation, and further studies are planned.

SUMMARY

A spontaneously occurring ovarian teratoma containing undifferentiated "embryonic" and many types of differentiated tissue is described.

The tumor was transplanted subcutaneously and grew in males and females of the strain in which it originated ($C3H_eB$), in the C3H strain, and in F_1 hybrids of C57BL/6 × C3H.

The transplanted tumors maintained the pleomorphic character of the original tumor through nine serial transplants. It is considered that the essential elements of this tumor are the undifferentiated cells which are pluripotent. In giving rise to the differentiated tissues, the undifferentiated cells showed their potentialities and gave proof of their pluripotency.

ACKNOWLEDGMENTS

We are indebted to Dr. Allen B. Griffen for the determination of diploidy of the chromosomes and for the mitotic counts.

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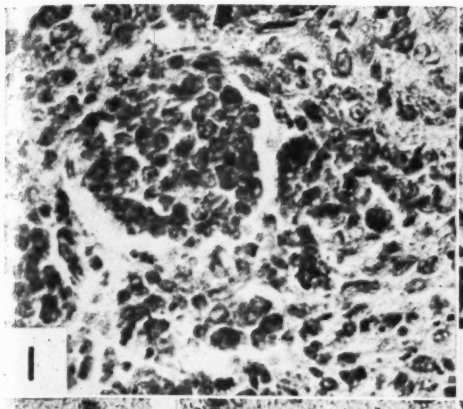

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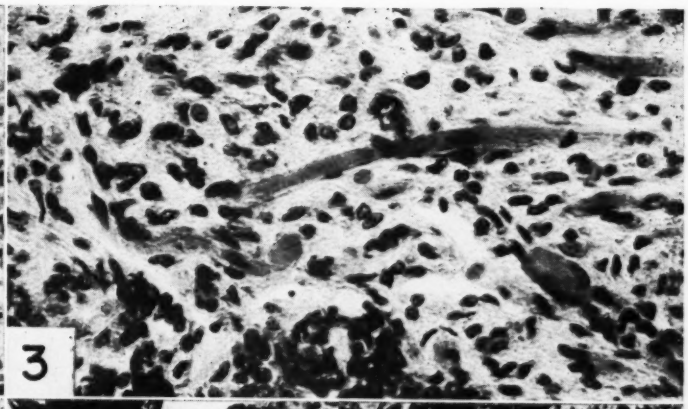

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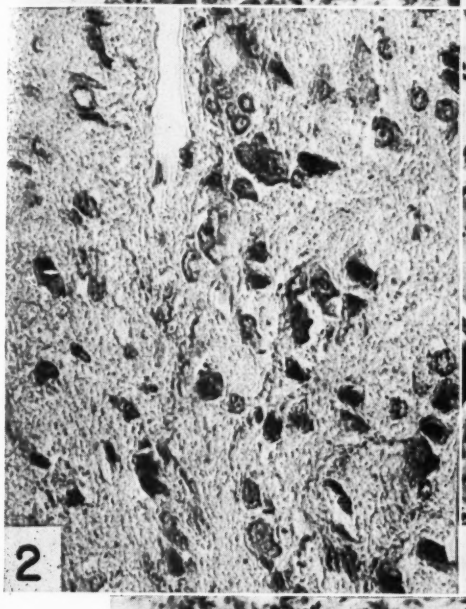

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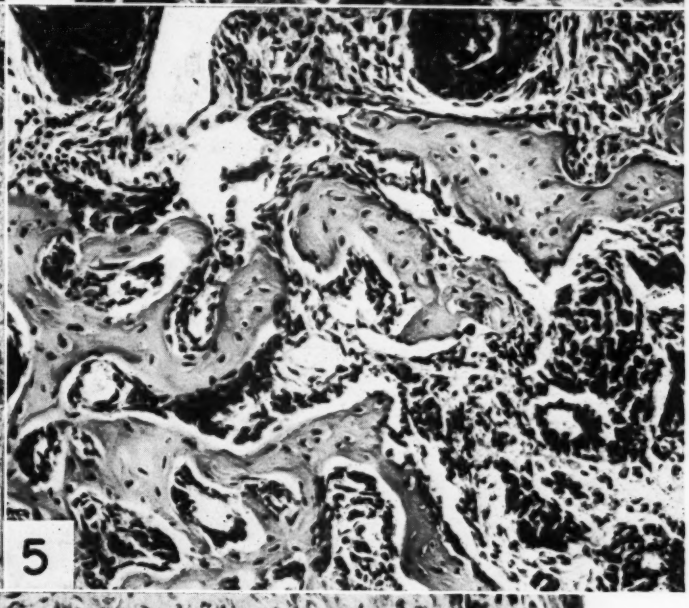

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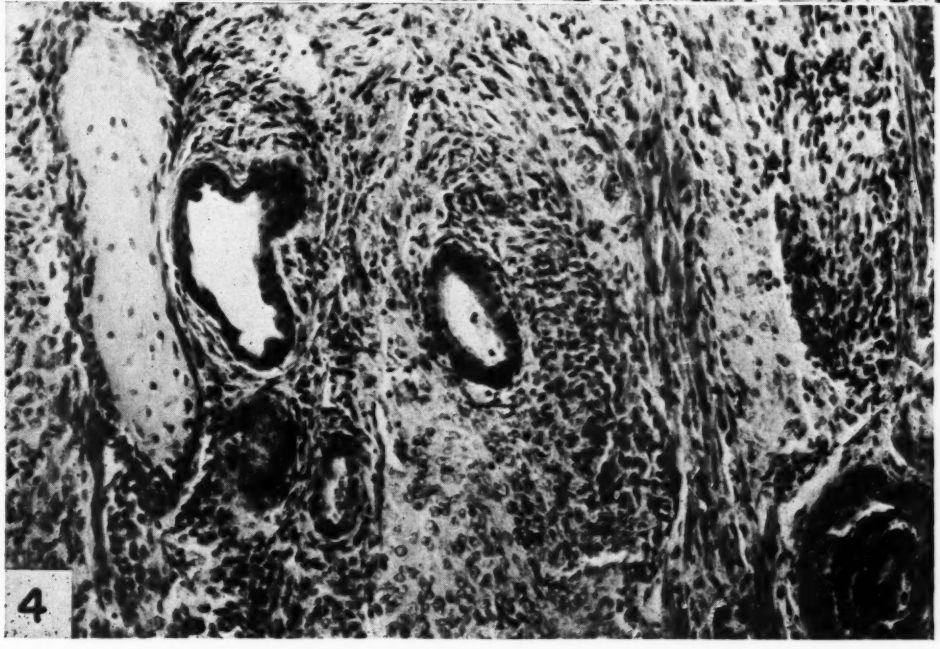

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All the sections were stained with hematoxylin and eosin except Figure 2.

FIG. 1.—The original ovarian teratoma showing undifferentiated round or oval-shaped cells arranged in a small nest. ×400.

FIG. 2.—Nervous tissue in the ovarian teratoma. Phosphotungstic acid stain. ×400.

FIG. 3.—Striated muscle fibers in the ovarian teratoma. ×400.

FIG. 4.—Tumor produced by the first transplant generation, showing bone, a cyst lined by ciliated epithelium, striated muscle, nervous tissue, and a cyst lined by pigmented stratified squamous epithelium. ×200.

FIG. 5.—Tumor produced by the second transplant generation showing bone with marrow spaces, stratified squamous epithelium and a few small alveoli. ×200.

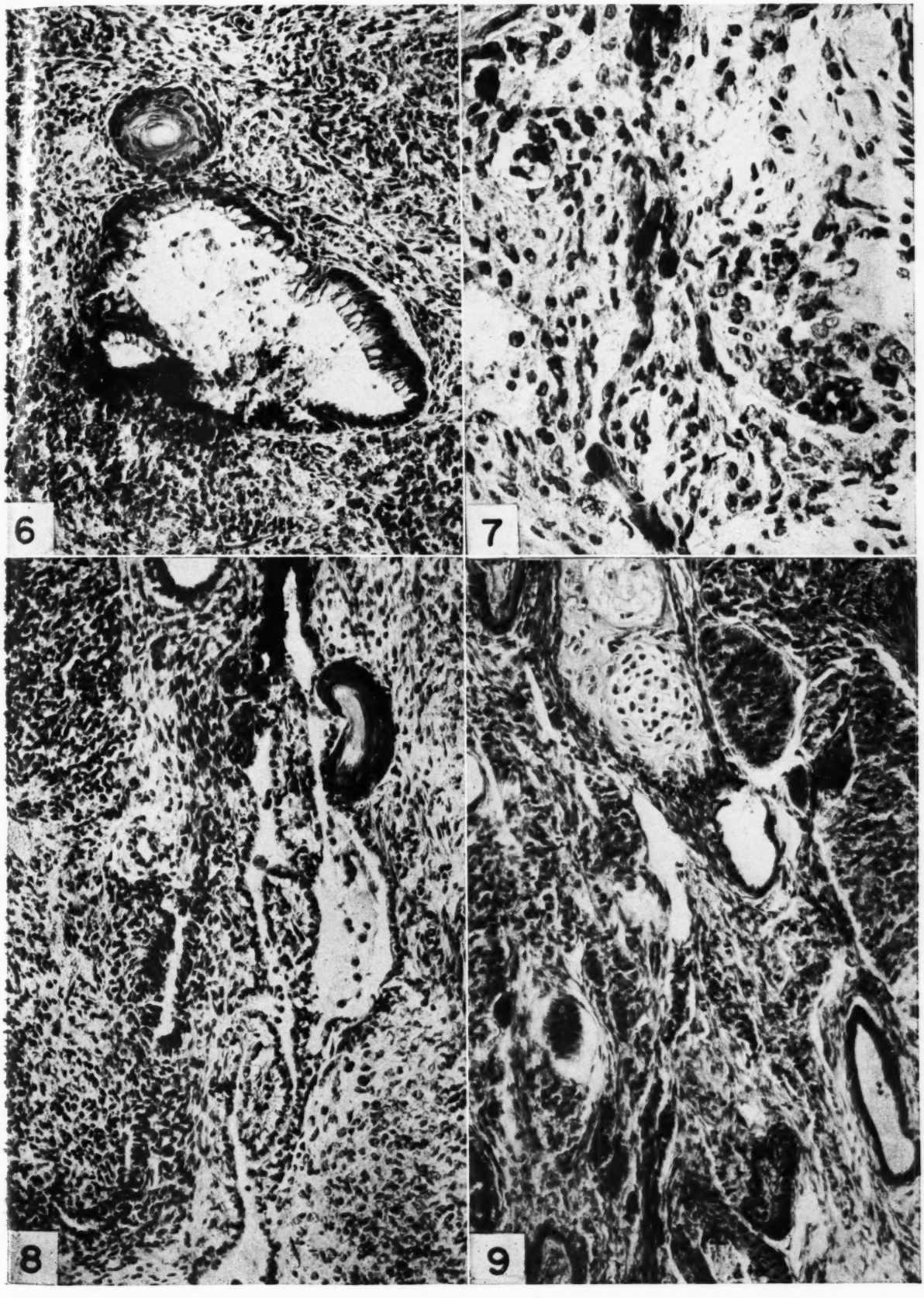
All the sections were stained with hematoxylin and eosin

FIG. 6.—Tumor produced by the fifth transplant generation showing a small cyst lined by stratified squamous epithelium and a large cyst lined by pigmented epithelial cells and goblet cells intermingled with columnar cells. ×200.

FIG. 7.—Striated muscle fibers in a tumor of the fifth transplant generation. ×400.

Fig. 8.—Tumor produced by the fifth transplant generation, showing a tube lined by pigmented columnar and stratified squamous epithelium and columnar epithelium intermingled with goblet cells. Finger-like structures resembling intestinal villi project into the tube. ×200.

FIG. 9.—Tumor produced by the sixth transplant generation. Cartilage, bone, cysts lined by cuboidal epithelium and other cysts lined by stratified squamous epithelium can be distinguished. ×200.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Protection against Radiation-induced Lymphoma Development by Shielding and Partial-Body Irradiation of Mice*

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It has been previously shown (5) that lymphoid tumors, which are readily induced by whole-body irradiation of C57 black mice, occur infrequently when the treatment is localized to an area of one-half the body or less or when a lead shield is placed over a peripheral region such as the thigh or lower extremity during irradiation. Although repeated irradiation of either half of the body alone is ineffective, alternate irradiation of the upper and lower halves of the body has yielded about the same lymphoma incidence as whole-body irradiation when the interval between exposure of the two halves of the body was 24 hours or less.

The present report is based upon two additional experiments concerned with these phenomena. In one experiment, the time interval between irradiation of alternate halves of the body has been progressively lengthened to determine the longevity of the effects produced in each half of the body. The other experiment was designed to determine whether the protective effect of peripheral shielding was proportional to the volume of tissue shielded and whether irradiation of the previously shielded volume would restore lymphoma incidence to the levels observed in whole-body irradiated mice.

METHODS

Experiment 1.—Strain C57 black mice of both sexes, aged 33 ± 3 days at the start of irradiation, were distributed among eight experimental groups. All groups were immobilized on wooden boards during irradiation and received a total of 673 r in four equally fractionated rounds of treatment.[1]

Each round of treatment involved the completion of irradiation to both halves of the body. The interval from the beginning of each round to the beginning of the next round was 8 days in all but one group. In this group (VI), the interval was extended by 3 days, with the result that successive rounds were started on days 1, 12, 23, and 34, instead of on days 1, 9, 17, and 25.

Six groups received alternate doses of irradiation to the upper and lower halves of the body; in any one group, animals in a given cage received their first treatment over the upper half, while those in the next cage received the first treatment over the lower half of the body. These groups differed only in the time required for completion of each round, as seen in Table 1. The 8-day group (VII) necessarily received twice the dose per exposure and half as many exposures, for an equal total dose.

A control group (I) received eight whole-body exposures of 86 r each at 96-hour intervals in a time sequence exactly comparable with Group IV, which received four alternate half-body treatments of 168 r each at 96-hour intervals. Volume dose in this control group was thus made equal to that of the alternate half-body treated groups. A final group (VIII) received four treatments to the upper half of the body on days 1, 2, 9, and 10, followed by treatment to the lower half of the body on days 17, 18, 25, and 26; this time sequence is comparable to the 24-hour interval for alternate half-body irradiation in Group II.

Experiment 2.—C57 black mice of both sexes were distributed among ten experimental groups and irradiated beginning at 33 ± 3 days of age. There were two groups of irradiated controls. In one, groups of eight mice were irradiated together in shallow lucite containers; mice in the other treated control group were immobilized on wooden boards and irradiated in groups of four to simulate the conditions of irradiation in the shielded groups. A total dose of 673 r was delivered in four equal treatments at 4-day intervals.[1]

In five of the shielded groups, the plan was to determine whether the volume of shielded tissue was proportional to the degree of protection. To this end, one group was shielded over the tail alone during irradiation; another group over the right thigh, with a lead strip 1 cm. in width; another group over the entire lower extremity; another over both lower extremities with the tail exposed; and another over the lower abdomen, with a lead strip 15 mm. wide. The lead shielding material in all instances was 3 mm. in thickness.

It also seemed of interest to determine whether irradiation of the previously shielded regions would abolish the protective effect. In one group the lower extremities and tail were shielded initially and then irradiated immediately afterward with the remainder of the body shielded. In another group the same procedure was used, but the interval between irradiation of the two regions was 24 hours. A final group had a different extremity shielded during each of the four treatments.

* This investigation was supported by a grant from the National Cancer Institute, National Institutes of Health, Public Health Service.

[1] Physical factors were: 120 kvP, 9 ma., 0.25 mm. Cu and 1.0 mm. Al added filter, 30 cm. target-mouse distance, output 32.2 r/min., HVL 0.39 mm. Cu.

Received for publication February 24, 1952.

In both experiments animals were maintained under identical laboratory conditions and received Purina Laboratory Chow and water ad libitum. Autopsies were routinely performed on moribund or dead animals, and the presence of lymphoid tumors was established histologically when gross examination left any doubt. Data below are based upon an observation period of approximately 300 days following the start of irradiation. Mean latent periods have been calculated from the first day of treatment.

RESULTS

Experiment 1.—The results are summarized in Table 1. There was an overwhelming response in the group which received split whole-body treatments at 96-hour intervals, and lymphoma incidence attained 100 per cent in this group at 267 days. Alternate half-body irradiation yielded a maximal incidence and minimal mean latent period when the interval between treatment of the two body halves was 24 hours. The incidence decreased, and the latent period increased slightly with increasing intervals thereafter through 96 hours. When the interval was further increased to 8 days, the final incidence dropped very considerably (Group VII). The response when the two body halves were treated at 96-hour intervals was not appreciably different when the interval between rounds of treatment was extended from 4 to 7 days (Groups V and VI), suggesting that the time required for complete exposure of the body is more critical than the time interval between rounds. Lymphoma development was almost completely inhibited when animals were irradiated first over the upper half of the body for four consecutive treatments and then similarly over the lower half of the body (Group VIII). The tumors which appeared in the alternate half-body irradiated groups were neither grossly nor histologically different from those induced in the whole-body irradiated animals.

Experiment 2.—Results are presented in Table 2, and cumulative incidence curves may be seen in Chart 1. Lymphoid tumor incidence was again high in the systemically irradiated groups and was not significantly different in the group which was immobilized during irradiation, indicating that immobilization alone does not account for the marked protective effect noted when animals are peripherally shielded.

There was again a striking inhibition of lymphoma development in animals shielded over any of the localized regions except the tail. No significant protection was observed in the group shielded over the tail alone (Group V). Lymphoma incidence was significantly lower in animals shielded over both lower extremities or the lower abdomen (Groups IX and X) than in those shielded over one lower extremity or the thigh alone (Groups VII and VIII), suggesting that there may be a relationship between the volume of tissue shielded and the degree of protection.[2]

When the lower extremities and tail were shielded initially and then irradiated either immediately thereafter (Group III) or at an interval of 24 hours (Group IV), the protective effect of shielding was abolished, and lymphoma incidence was not significantly different from that in the whole-body irradiated groups. Similarly, there was no evidence of protection when a different extremity was shielded at each of the four successive exposures (Group VI), indicating that the dose delivered to the extremities under these conditions was still capable of inactivating the protective mechanism.

[2] Comparison of the pooled data of Groups VII and VIII against the pooled data of Groups IX and X in the form of a 2×2 contingency table yielded a value of χ^2, with one degree of freedom, of 10.324, and an associated probability very slightly greater than 0.001 (1).

TABLE 1

EFFECT OF ALTERNATE HALF-BODY IRRADIATION ON DEVELOPMENT OF LYMPHOID TUMORS IN C57 BLACK MICE

Group	Technic of individual round of irradiation	Day of treatment: 1st round	2d round	3d round	4th round	X-ray dose	No. of mice	Mice with lymphomas: No.	Per cent	Latent period av.+range (days)	No. of mice dead (neg.)	No. of mice alive
I	Whole-body treatment at 96-hour interval	1+5	9+13	17+21	25+29	84 r×8=673 r	43	43	100	162 (110–267)	0	0
II	Alternate halves at 24-hour interval	1+2	9+10	17+18	25+26	168 r×4=673 r (each half)	46	32	70	184 (116–247)	2	12
III	Alternate halves at 48-hour interval	1+3	9+11	17+19	25+27	168 r×4=673 r (each half)	46	34	74	186 (117–296)	2	10
IV	Alternate halves at 72-hour interval	1+4	9+12	17+20	25+28	168 r×4=673 r (each half)	45	27	60	190 (96–268)	2	16
V	Alternate halves at 96-hour interval	1+5	9+13	17+21	25+29	168 r×4=673 r (each half)	44	25	57	196 (135–319)	3	16
VI	Alternate halves at 96-hour interval	1+5	12+16	23+27	34+38	168 r×4=673 r (each half)	45	21	47	217 (123–338)	1	23
VII	Alternate halves at 8-day interval	1	9	17	25	336 r×2=673 r (each half)	46	6	13	213 (155–281)	2	38
VIII	Upper half×4, at 24-hour interval	upper		lower		168 r×4=673 r (each half)	40	2	5	237 (197–277)	1	37
	Lower half×4	1+2	9+10	17+18	25+26							

The lymphoid tumors in all groups resembled those previously described after whole-body irradiation and tended predominantly to involve the thymus, as previously observed (4). It is noteworthy, therefore, that in the four locally shielded groups in which striking protection was observed, the thymic area was in no way protected by the shielding and received the same dose as in the systemically irradiated groups.

DISCUSSION

These experiments provide further evidence concerning the nature of the indirect mechanism involved in lymphoid tumor induction in irradiated mice. In both experiments, it can be seen that, when two large regions of the body are alternately irradiated in such a manner as to simulate total-body irradiation and when the alternate treatments occur within a suitable time interval, the yield of lymphomas is high. When certain regions of the body are shielded, or when the lower half of the body remains untreated while the upper half is repeatedly irradiated, few lymphomas occur. This suggests that intact, nonirradiated tissues can inactivate, at least in part, the leukemogenic effects of x-rays produced within the remainder of the body. There appears to be at least a crude relationship between the quantity of tissue shielded and the degree of protection.

In an attempt to explain the inhibitory effect of peripheral shielding, it was previously postulated that a "humoral" mechanism might be involved (5). The view was expressed that irradiated tissue might release some agent into the circulation, the action of which upon the radiation-damaged thymus is necessary for lymphoma induction. It was further postulated that nonirradiated tissue might retain the capacity to destroy such a circulating agent. The converse possibility, that intact tissue normally produces a protective agent whose manufacture is interfered with by irradiation, was also entertained. The above concepts are in need of some modification in the light of present evidence. It still seems likely that a blood-borne influence is involved, but there seems to be no reason why this may not be of cellular nature, rather than truly humoral.

The inhibitory effect of leg shielding appears distinctly analogous to the protection exerted by spleen shielding on acute radiation mortality in mice (3). More recent experiments point to a distinct protective effect of bone marrow transfusions on radiation mortality in mice, guinea pigs, and rabbits (2, 6). It has not yet been established whether these phenomena are due to the release of a humoral substance or to recolonization of the radiation-damaged hematopoietic tissues by the release of actively proliferating hematopoietic cells from the shielded spleen or the injected bone marrow suspensions. The alternate possibility must therefore be considered that, in Experiment 2, leg shielding resulted in protection of bone marrow in the shielded area, leaving a reservoir of healthy

TABLE 2

EFFECT OF SHIELDING ON DEVELOPMENT OF RADIATION-INDUCED LYMPHOMAS IN C57 BLACK MICE

Group	Area shielded	X-ray dose	No. of mice	Mice with lymphomas			No. of mice dead (neg.)	No. of mice alive
				No.	Per cent	Latent period av. range (days)		
I	None	168 r×4=673 r every 4 days	55	48	87	144 (78–210)	1	6
II	None; immobilized during x-ray	168 r×4=673 r every 4 days	53	41	77	162 (103–228)	1	11
III	Both lower extremities and tail; then body	168 r×4=673 r (to each part) every 4 days	57	47	82	152 (98–238)	1	9
IV	Both lower extremities and tail; 24-hour interval then body	168 r×4=673 r (to each part) every 4 days	52	45	87	157 (99–233)	0	7
V	Tail	168 r×4=673 r every 4 days	58	48	83	161 (77–240)	0	10
VI	Different extremity each treatment	168 r×4=673 r every 4 days	53	43	81	163	0	10
VII	One hind thigh	168 r×4=673 r every 4 days	55	13	24	178 (135–268)	1	41
VIII	One lower extremity	168 r×4=673 r every 4 days	58	15	26	169 (114–249)	1	42
IX	Both lower extremities	168 r×4=673 r every 4 days	57	5	9	173 (124–279)	1	51
X	Lower abdomen	168 r×4=673 r every 4 days	57	5	9	177 (145–214)	0	52

hematopoietic cells which, by hematogenous dissemination, subsequently recolonized the thymus, spleen, and other irradiated tissues. The failure of tail shielding to inhibit lymphoma development seems difficult to explain on the basis of the release or inactivation of a humoral agent. There is, however, evidence that bone marrow in the tail of the mouse is largely inactive and fatty,[3] and this lack of response might be explained by the absence of a hematopoietic reservoir in the tail which could give rise to cellular metastasis and accelerated thymic recovery.

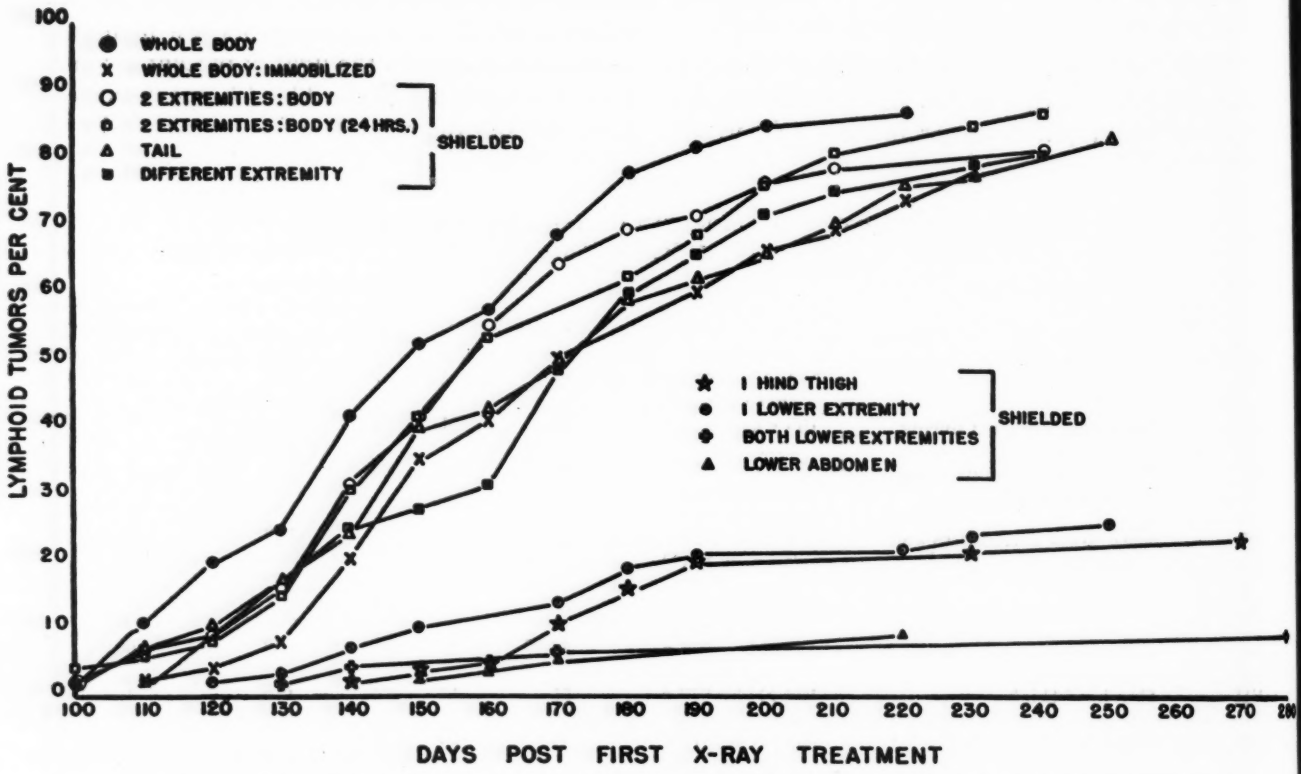

CHART 1.—Effect of shielding on development of radiation-induced lymphomas in C57 black mice

SUMMARY

Experiment 1.—Alternate half-body irradiation of C57 black mice yielded a maximal lymphoma incidence when the interval between treatment of the two body halves was 24 hours and decreased gradually thereafter through 8 days. Response was largely abolished when animals were irradiated first over the upper half of the body for four consecutive treatments and then similarly over the lower half of the body.

Experiment 2.—Lymphoma incidence was strikingly reduced by shielding the thigh, lower extremity, or lower abdomen, but not by shielding of the tail during irradiation. There was a suggestive relationship between the volume of tissue shielded and the degree of protection. Incidence was restored to normal high levels when the lower extremities were shielded initially and then locally irradiated either immediately or at an interval of 24 hours. There was no inhibition when a different extremity was shielded at each of four successive exposures.

[3] E. P. Cronkite, personal communication.

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Testosterone Prevention of Post-Irradiation Lymphomas in C57 Black Mice*

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It was recently reported that testosterone strikingly inhibits radiation-induced lymphoid tumor development in intact and castrate adult male C57 black mice (5). This result is consistent with the earlier observations of Murphy (9) on spontaneous leukemia and of Gardner (1) in irradiated female BC mice.

The present report is concerned with two additional experiments on C57 black mice. In one, the inhibitory effect of testosterone was observed only when it was given concurrently with irradiation. In the other, the degree of inhibition has been shown to be essentially the same in female mice, as previously reported for males of this strain, though somewhat greater in spayed than in intact animals.

METHODS

Experiment 1.—Intact male C57 black mice were distributed among seven experimental groups and given whole-body irradiation to a total dose of 673 r in four equally fractionated treatments, at intervals of 4 days, starting at 60 days of age.[1] Six groups received intramuscular injections of 0.5 mg. testosterone in 0.02 cc. of stabilized aqueous suspension twice weekly for 5 weeks.[2] The testosterone-injected groups differed only in the time at which testosterone injections were started in relation to the time of irradiation, as indicated in Table 1. The seventh group received similarly timed placebo injections of the suspension medium alone, starting concurrently with the first irradiation and continued for 5 weeks.

Experiment 2.—Litter-mate female C57 black mice were distributed among four groups at the time of weaning. Groups II and IV were spayed at 2 months of age. The intact and spayed groups then received the same dose of fractionated whole-body irradiation as in Experiment 1, beginning 2–4 days postoperatively. Groups III and IV received testosterone injections intramuscularly twice weekly for 10 weeks, the dose of testosterone and volume of injection being the same as in Experiment 1. Groups I and II received placebo injections of the suspension medium alone for 10 weeks. All injections were started immediately after the first x-ray treatment.

In both experiments, all animals were identically caged and maintained on Purina Laboratory Chow and water ad libitum. Mice that died of any cause before the time of appearance of the first lymphoid tumor have been omitted. All animals were carefully examined at autopsy. In most instances the diagnosis of a mediastinal or disseminated lymphoma could be made on gross examination, but diagnoses were established histologically in all questionable instances.

RESULTS

Experiment 1.—The data are summarized in Table 1, and cumulative incidence curves are seen in Chart 1. There was a striking inhibition of lymphoma development in Group II, in which testosterone injections were started concurrently with irradiation. There was no protection when testosterone injections were started at 1 month of age and completed by the time irradiation began. There was a slight increase in latent period when testosterone was not started until 3 weeks after the first x-ray treatment, but the ultimate incidence in this group did not differ significantly from placebo-injected irradiated controls. No inhibition was observed when testosterone was started 6–12 weeks after irradiation.

Experiment 2.—The data are similarly presented in Table 2. Testosterone yielded a pronounced inhibition of lymphoid tumor development in both intact and spayed female mice. The degree of inhibition was somewhat greater in the spayed than in the intact group, although the differences are of borderline significance at the 0.05 level. The degree of inhibition in the spayed group was about the same as that previously noted in male mice treated with testosterone.

DISCUSSION

Cortisone has been shown to inhibit lymphoma development significantly when started as late as 6 weeks after the completion of x-radiation (6). In contrast, the data of Experiment 1 indicate that testosterone is effective only when given concurrently with irradiation. This suggests that testosterone and cortisone exert their inhibitory effects upon the leukemogenic process independently of one another. Further studies on the acute effects of both agents upon irradiated lymphoid tissues are needed.

It is of interest that pretreatment with testosterone at 1 month of age did not decrease

* This investigation was supported by a grant from the National Cancer Institute, National Institutes of Health, Public Health Service.

[1] Physical factors were: 120 kvP, 9 ma., 0.25 mm. Cu, and 1.0 mm. Al added filter, HVL. 0.36 mm. Cu, target-mouse distance 30 inches, output 32.2 r/min.

[2] Aqueous suspensions of testosterone, 25 mg/cc, stabilized with 0.35 per cent aluminum phosphate, and placebo vials of 0.35 aluminum phosphate suspension were generously supplied by Dr. Edward Henderson of the Schering Corporation, Bloomfield, N.J.

Received for publication February 24, 1952.

TABLE 1

Time of Testosterone Injection and Lymphoma Development in Irradiated C57 Black Male Mice

Group	Material injected	Injections started	No. of mice	Mice with lymphomas			No. dead (neg.)*	No. alive*
				No.	Per cent	Mean latent period (days)		
I	Placebo	simultaneously with x-ray	28	20	71	176	2	6
II	Testosterone	simultaneously with x-ray	30	6	20	218		24
III	Testosterone	4 weeks before x-ray	29	21	72	161	1	7
IV	Testosterone	3 weeks after first x-ray	30	19	63	193	6	5
V	Testosterone	6 weeks after first x-ray	30	26	87	189	1	3
VI	Testosterone	9 weeks after first x-ray	31	24	77	199	6	1
VII	Testosterone	12 weeks after first x-ray	32	27	84	170	2	3

* 300 days after first x-ray.

TABLE 2

Effect of Testosterone Injections on Radiation-induced Tumors in C57 Black Female Mice

Group		Material injected	No. of mice	Mice with lymphomas			No. dead (neg.)*	No. alive*
				No.	Per cent	Mean latent period (days)		
I	Intact	Placebo	30	27	93	155	3	0
II	Spayed	Placebo	25	23	92	170	2	2
III	Intact	Testosterone	28	7	17	270	16	5
IV	Spayed	Testosterone	27	2	4	311	15	10

* 300 days after first x-ray.

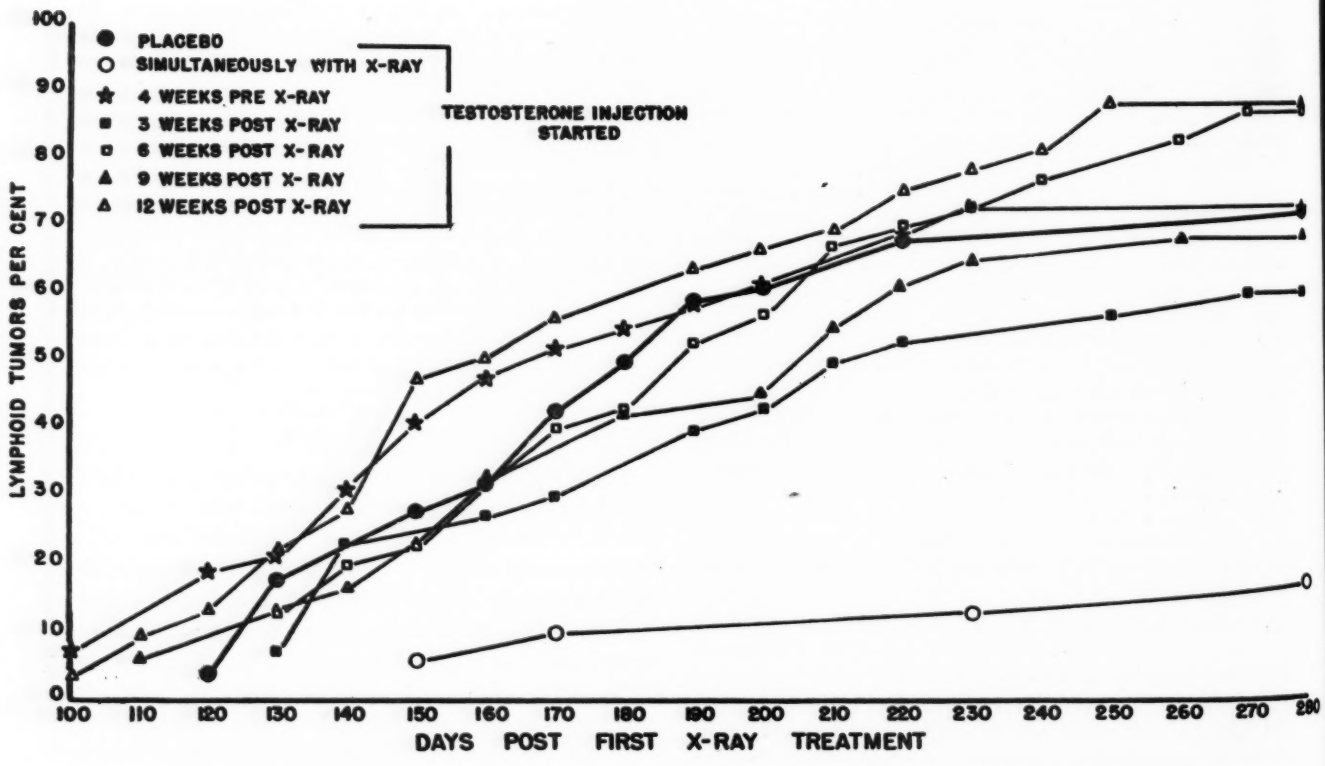

Chart 1.—Time of testosterone injection and lymphoma development in irradiated C57 black male mice

lymphoma incidence, since testosterone provokes a striking and selective thymic involution which might, on a priori grounds, have been considered analogous to thymectomy. It has previously been shown that thymectomy prior to irradiation virtually abolishes lymphoma development in C57 black mice (4). From this result, it would appear that the size of the thymus at the time of irradiation is unrelated to leukemogenic susceptibility.

Testosterone is also an effective inhibitor of lymphoma development in female mice. The apparently greater degree of suppression exerted in spayed females is of considerable interest, if confirmed by subsequent experiments, because it is the first suggestion that *endogenous* estrogen secretion may contribute to leukemogenic susceptibility in mice of this strain. Estrogens are well known to be leukemogenic for some strains (3), and Kirschbaum, Shapiro, and Mixer (8) have previously shown that *exogenous* estrogen may act synergistically with irradiation in eliciting thymic lymphomas in a susceptible strain. However, strain C57 black mice tolerate exogenous estrogens poorly, and estrogens do not appear to be leukemogenic for this strain (2, 7). Moreover, the incidence of lymphomas in irradiated females, whether immature (4) or adult,[3] is not altered by gonadectomy. The present experiment suggests that spayed females are more susceptible to the lymphoma-inhibiting action of testosterone; that this may be due in part to the "neutralization" of endogenous estrogen is a plausible explanation deserving further experimental study.

SUMMARY

In two experiments concerned with radiation-induced lymphoid tumors of C57 black mice, it has been shown that: (*a*) testosterone inhibits lymphoma development only when given concurrently with irradiation and (*b*) the inhibitory effect of testosterone previously noted in male mice of this strain is also observed in females, the degree of inhibition being apparently somewhat greater in spayed than in intact animals.

[3] Unpublished data.

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Effect of Pituitary Growth Hormone in Mice*

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The carcinogenic and growth-promoting effects of long-term administration of pituitary growth hormone in normal adult female rats have been reported previously (1, 3, 4, 5). The present report is concerned with the effects of large amounts of pituitary growth hormone in young mice of several different strains injected for a maximum period of 194 days.

MATERIALS AND METHODS

Mice of strains A, C3H and C57 black, 3–4 months old, were used as the experimental animals. In strains A and C3H, ten males and ten females were injected with physico-chemically pure pituitary growth hormone;[1] identical numbers of male and female mice were used as uninjected controls. In the C57 black strain, five males and five females were injected with growth hormone; the same number of uninjected mice of each sex were used as controls. All experimental animals were injected intraperitoneally daily for 6 days each week for a maximum period of 194 days. The initial daily dosage of growth hormone was 0.04 mg.; this was increased at intervals to a maximum dosage of 2.0 mg. daily. This dosage of growth hormone is very high in relation to the body weight—approximately 20 times that used in female rats in which tumors occurred (1, 3, 4, 5) (Chart 1). The animals were weighed every 5 days. At autopsy all organs were examined under a dissecting binocular microscope, removed, weighed, and fixed in formalin. Representative sections of all tissues were taken for microscopic study.

OBSERVATIONS

During the early stages of the experiment the rate of growth of the mice receiving growth hormone did not differ from that of their controls. As the dosage of growth hormone was increased, the female mice of all three strains showed a greater gain in weight than their controls (Chart 1).

At autopsy, both the relative and absolute increases in body weight of female mice injected with growth hormone were considerably greater than those of their controls (Table 1). The male mice of all three strains receiving growth hormone showed no significant alteration in their growth curve, as compared to that of the controls. The average total body length for both male and female mice of all three strains was slightly greater in the mice receiving growth hormone than in their respective controls. The hearts, lymph nodes, spleens, livers, kidneys, and adrenal medullas of the injected animals were larger than those of the controls.

As shown in detail below *there was no increase in the incidence of neoplasms in the three strains of mice injected with growth hormone.*

Strain A male mice.—There were no neoplasms in either the experimental mice or their controls. The hearts, lymph nodes, spleens, and kidneys of the mice injected with growth hormone were larger than those of the controls and roughly proportional to the total body weight. The livers also were larger than those of the controls (Table 2), and the hepatic cells were hypertrophied and exhibited greater nuclear pleomorphism and hyperchromatism. The adrenal glands of the injected animals showed medullary hypertrophy.

Strain A female mice.—There were no neoplasms in the mice injected with growth hormone. In one of the controls there was pulmonary adenomatosis. The hearts, lymph nodes, spleens, and kidneys of the animals receiving growth hormone were hypertrophied. The livers of these animals showed changes similar to those noted in the males. The adrenal medullae were hypertrophied in the injected mice.

Strain C3H male mice.—No neoplasms occurred in either the mice injected with growth hormone or their controls. The hearts, lymph nodes, spleens, and kidneys of the mice receiving growth hormone were hypertrophied. The livers were also enlarged; the hepatic cells showed greater pleomorphism than those of the controls. The adrenal medullae were hypertrophied in the injected group.

* Aided by grants from the U.S. Public Health Service (RG-409 and C-1098), the Research Board of the University of California (654), University of California Cancer Grant, and the Albert and Mary Lasker Foundation.

[1] The mice used in these experiments were kindly supplied by Dr. Michael B. Shimkin of the Laboratory of Oncology, University of California Medical School, San Francisco.

Received for publication February 25, 1952.

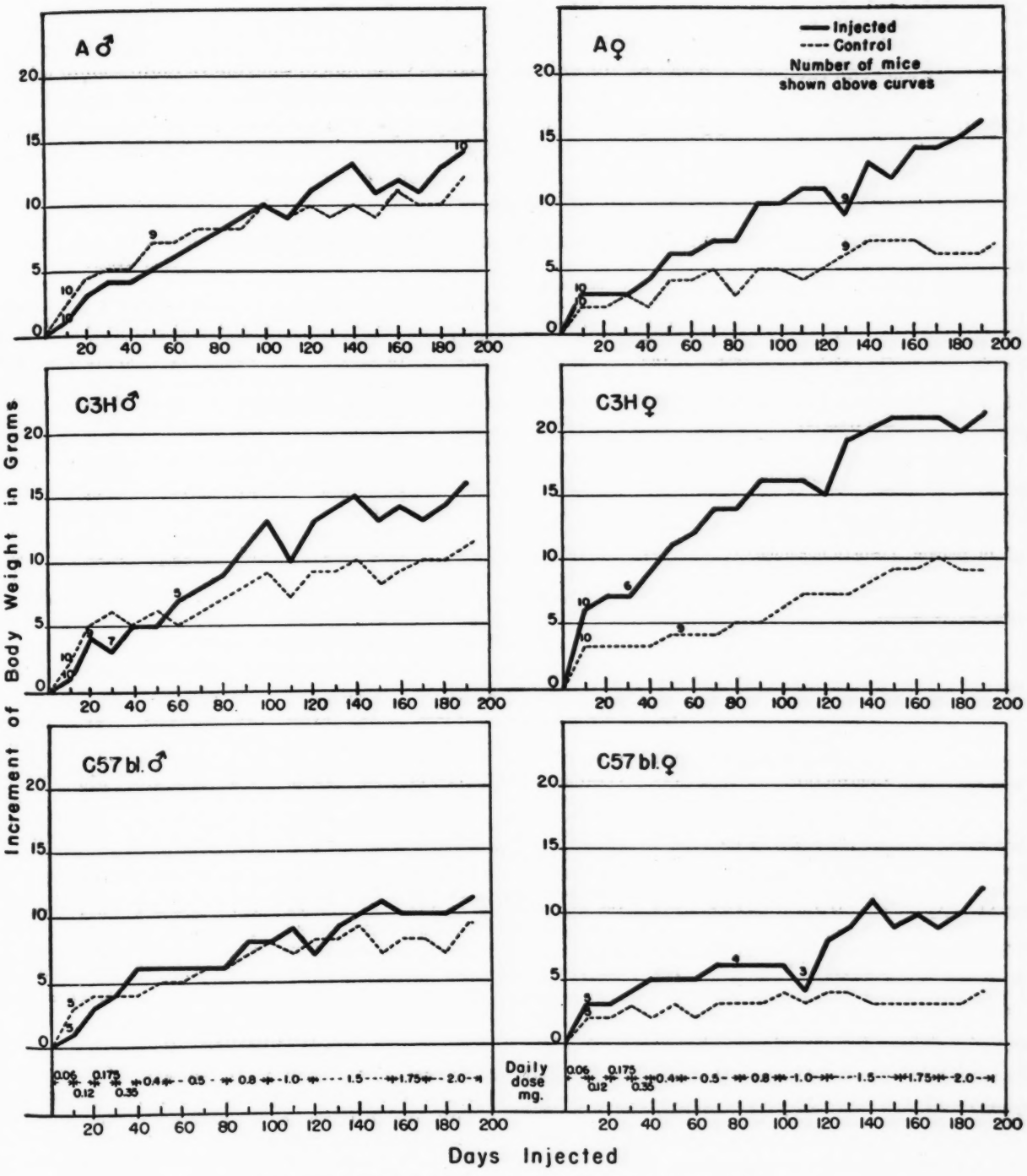

CHART 1.—Effect of growth hormone on body weight of mice

Strain C3H female mice.—Mammary carcinomas occurred in two mice receiving growth hormone and in two controls. The hearts, lymph nodes, spleens, and kidneys of the mice injected with growth hormone were hypertrophied. The livers were also enlarged and showed cellular pleomorphism to a greater degree than the controls. The adrenal medullae were hypertrophied in the experimental group.

Strain C57 black male mice.—There were no tumors in either the experimental or control mice. The mice injected with growth hormone showed hypertrophy of hearts, lymph nodes, spleens, and livers. The hepatic cells were hypertrophied; there was moderate nuclear pleomorphism. The adrenal medullae were larger in the experimental mice than in the controls.

Strain C57 black female mice.—There were no tumors in either the experimental mice or their controls. The hearts, lymph nodes, spleens, livers, kidneys, and adrenal medullae were hypertrophied in the experimental group.

SUMMARY

The administration of pituitary growth hormone to young male and female mice of the A, C3H, and C57 black strains did not cause an increased incidence of tumors. The amount of growth hormone in relation to body weight administered to these mice was extremely high. The efficacy of growth hormone in these mice was shown by the greater gain in body weight in females and by visceral enlargement.

TABLE 1

EFFECT OF PITUITARY GROWTH HORMONE ON LENGTH AND BODY WEIGHT OF MICE

Mice	Treatment	Days injected	Maximum age at autopsy (days)	Total length at autopsy Av. (cm.)	Total length at autopsy Range (cm.)	Body weight Autopsy (gm.)	Body weight Gain Av. (gm.)	Body weight Gain Range (gm.)	Body weight Gain Per cent gain
A male	Growth hormone (9)*	193	282	20.6	19.1–21.4	37	14	10–16	58
	Controls (9)*		282	19.1	17.9–19.7	31	12	9–17	63
A female	Growth hormone (9)*	194	290	20.1	19.1–21.2	37	17	12–23	81
	Controls (9)*		290	18.4	17.3–19.5	27	9	6–13	50
C3H male	Growth hormone (5)*	193	372	20.8	20.5–21.2	41	15	12–19	57
	Controls (10)*		372	20.0	19.6–21.0	36	11	6–13	44
C3H female	Growth hormone (6)*	193	244	20.3	19.6–21.0	40	23	20–30	135
	Controls (6)*		291	18.8	18.0–19.6	31	12	8–13	63
C57 bl. male	Growth hormone (5)*	194	266	19.2	19.0–19.5	33	12	10–14	58
	Controls (5)*		266	18.1	17.7–18.4	27	9	7–11	50
C57 bl. female	Growth hormone (3)*	194	291	19.0	18.6–19.4	31	12	8–16	63
	Controls (5)*		291	17.4	16.9–18.4	23	5	2– 7	21

* The figures in parentheses show the number of mice which survived for the total duration of the experiment and were used for determining the average measurements.

TABLE 2

EFFECT OF GROWTH HORMONE ON VISCERA OF MICE

Mice	Treatment	Heart Av. (mg.)	Heart Range (mg.)	Spleen Av. (mg.)	Spleen Range (mg.)	Thymus Av. (mg.)	Thymus Range (mg.)	Liver Av. (gm.)	Liver Range (gm.)	Kidneys Av. (mg.)	Kidneys Range (mg.)
A male	Growth hormone (9)*	164	134–201	183	110–304	18	13–24	2.2	1.6–2.8	603	375–764
	Control (9)*	143	122–158	129	77–174	19	14–25	1.6	1.3–2.0	518	380–518
A female	Growth hormone (9)*	148	122–204	169	135–209	19	8–31	2.3	1.8–2.7	561	435–660
	Control (9)*	118	94–140	137	58–228	23	18–43	1.6	0.9–1.9	450	295–528
C3H male	Growth hormone (5)*	166	148–197	153	139–190	17	12–26	2.5	2.2–2.9	755	700–896
	Control (10)*	145	124–168	117	83–140	18	16–22	2.0	1.7–2.3	655	600–741
C3H female	Growth hormone (6)*	165	89–219	239	160–369	27	24–30	2.5	2.2–3.0	586	464–642
	Control (6)*	135	127–154	140	97–168	25	21–30	1.7	1.4–1.9	460	400–507
C57 bl. male	Growth hormone (5)*	188	131–215	121	98–144	18	8–34	2.1	1.9–2.3	573	477–651
	Control (5)*	126	110–145	75	58– 94	25	18–32	1.4	1.3–1.6	379	319–427
C57 bl. female	Growth hormone (3)*	209	168–232	147	110–170	23	16–28	2.5	2.3–2.7	539	500–601
	Control (5)*	136	106–174	76	44– 92	25	14–40	1.4	1.1–1.6	336	260–392

* The figures in parentheses show the number of mice which survived for the total duration of the experiment and were used in determining the average measurements.

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The Effect of Ultraviolet Radiation on the Production of Spontaneous Mammary Tumors in C3H Mice*

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Previous experiments carried out in a set of specially designed animal rooms with temperature and light control revealed a marked difference in tumor incidence between mice kept entirely in the dark and those exposed to artificial daylight, when the tumors were induced by painting with solutions of carcinogenic hydrocarbons. In both Swiss albino and C57 black mice there was a delay in the appearance of epidermoid carcinomas of the skin and a diminution in the number of animals which developed tumor in the groups exposed to light (7, 8). Light enhanced the leukemogenic properties of 3,4-benzpyrene and 20-methylcholanthrene in mice of the DBA strain (9).

It was decided to study mice subject to spontaneous mammary tumors under the same environmental conditions used previously and also to expose part of the group to ultraviolet radiation below the level necessary to produce skin cancer (5). It seemed possible that ultraviolet radiation, which is itself a carcinogenic agent when intense enough, might activate the tumor agent and increase the rate of tumor incidence. Apperly and Cary (2, 3), using strain A mice in one experiment and DBA mice (subline I) in another, found that exposure to ultraviolet radiation had a deterrent effect upon the incidence of spontaneous mammary cancer; but these experiments did not seem entirely conclusive.

EXPERIMENTAL PROCEDURE

The animal rooms were fully described in a previous publication (7). The banks of fluorescent lamps gave an even illumination of 300 foot-candles for 12 hours a day. This illumination was originally tested as giving a total energy of the order of 0.1 gm/cal/cm²/min. The temperature was automatically controlled, and the rooms were kept at 22° C.

The mice used were individually identified virgin females of strain C3H, Andervont subline. Shimkin (10) reported a mammary cancer incidence in such subjects of 97.4 per cent at a mean age of 10.4 months in 1945, while Andervont (1) in 1949 found carcinomas of the breast in 92.9 per cent at an average age of 11.3 months. All mice were kept in individual wire mesh cages and fed Purina Dog Chow and water ad libitum. Littermates were distributed evenly among the experimental groups, which were also carefully balanced for weight and age.

Ultraviolet radiation including the carcinogenic wave lengths (5) was given by a quartz mercury arc (General Electric Uviarc) at 21 inches. At this distance an exposure of 2.5 minutes produced a minimal erythema on human skin. When radiated, the mice were placed in small individual wide wire mesh cages in which a wire mesh trap kept the animals at an approximately constant distance from the light source. They were able to move about, however, during the exposure.

All animals were weighed once a month. There was no significant difference in the weights in different groups. The mice from the light room ate less and were less active.

The first definite appearance of the tumors was noted. When a tumor had grown to a moderate size the animal was killed, and the tumor was removed, sectioned, and examined microscopically. As the mammary tissue is wide spread, tumors appeared in many different locations—axilla, groin, back of neck, jaw, base of tail, etc. The histologic appearance of the carcinomas conformed to the types described by Dunn (6), which occur regularly in mice of the C3H strain.

RESULTS

Three separate experiments were carried out.

Experiment 1.—C3H mice of the Andervont subline, descendants of those obtained originally from Dr. H. B. Andervont and bred in Rochester, were used. These mice were divided into three groups. Group 1 was kept entirely in the dark. Group 2 was also kept entirely in the dark but was exposed to ultraviolet radiation, 2 erythema doses (E.D.) 3 times a week, which was increased to 3 E.D. 3 times a week, with a total dose ranging from 80 E.D. to 200 E.D. Group 3 was kept in the light room where the animals were exposed for 12 hours a day to visible light (300 foot-candles) which contained no ultraviolet rays.

There was a marked difference in the tumor incidence in the three groups (Table 1). At the age of 300 days, 60 per cent of the ultraviolet (U.V.) group had developed tumors, 33 per cent of the dark group, and none of the light group. The average age at which neoplasms appeared was 299 days in the U.V. group, 340 days in the dark group, and 394 days in the light group.

Experiment 2.—The second experiment exactly repeated Experiment 1, except that the C3H mice of the Andervont subline were obtained from the Roscoe B. Jackson Memorial Laboratory. The mice were all between 4 and 6 weeks old when received, and radiation was started at once in the ultraviolet group. The radiation was begun with one erythema dose 3 times a week and was increased to 3 E.D. 3 times a week. The total dose in all cases was 125 E.D. A few mice became ill from the radiation, which was perhaps begun too early, but 23 lived to tumor age (Table 2).

* Aided by a grant from the Jane Coffin Childs Memorial Fund for Medical Research.

Received for publication March 8, 1952.

The experiment was terminated when 100 per cent of the U.V. group had developed tumors and the surviving mice in the other two groups were 535 days old. The average tumor age did not show a significant difference, probably because the experiment was not continued until all animals developed tumors. The average age at onset of cancer was 347 days for the U.V. group, 351 days for the dark group, and 377 days for the light group.

Up to the age of 300 days there was no difference in tumor incidence in the three groups, but after that the rate of incidence in the U.V. group increased compared to the other two groups (Table 2).

TABLE 1

CUMULATIVE NUMBER OF MICE WITH MAMMARY TUMORS (Exp. 1)

Age (days)	Dark (12)	Dark and U.V. (15)	Light (16)
250	1	3	0
300	4	9	0
350	6	12	4
400	8	14	8
450	12	15	12
500			15
564			16

TABLE 2

CUMULATIVE NUMBER OF MICE WITH MAMMARY TUMORS (Exp. 2)

Age (days)	Dark (21)	Dark and U.V. (23)	Light (24)
250	1	2	3
300	4	5	4
350	9	13	9
400	14	19	13
450	18	22	19
535	18	23	21

As in Experiment 1, the mice given ultraviolet radiation developed tumors earlier. The difference between the dark and light groups which seemed significant in Experiment 1 was not apparent in Experiment 2.

Experiment 3.—In this experiment, C3H mice of the Andervont subline bred in Rochester were used, but the groups were set up differently. Group 1 was kept in the dark, and all other groups were exposed to visible light 12 hours a day. Group 2 had light plus exposure to ultraviolet radiation given 3 times a week beginning with 2 E.D. a day and increasing to 4 E.D. a day with the total dose ranging from 100 to 250 E.D. Group 3 was exposed to visible light only.

As the ultraviolet-irradiated animals were living in the light 12 hours a day instead of in the dark as in Experiment 1 and Experiment 2, the results of the three experiments may not be entirely comparable. The average age at the onset of the tumor was 340 days for the light plus U.V. group, 408 days for the light group, and 423 days for the dark group (Table 3).

A comparison of the results for the animals from all three experiments, at the age of 365 days, is given in Table 4. It is evident that the mice used in Experiment 3 differed from those in Experiments 1 and 2, as the tumors developed more slowly.

DISCUSSION

No consistent difference could be demonstrated at any given time in the relative frequency of mammary tumors between groups of mice housed in a light or dark environment in the absence of ultraviolet rays. Application of the chi-square test to the pooled total experience in light and dark failed to disclose any statistically significant differences in relative frequency of carcinoma of the breast at any interval between 300 and 600 days.

Mice that received ultraviolet irradiation developed their mammary cancers earlier than did the other groups in each experiment. The data obtained from all groups exposed to ultraviolet rays were compared to the total experience obtained in visible light and in the dark and to the two latter groups together, since no valid difference in the relative frequency of mammary tumors was demonstrated between them. Each of the three procedures gave the same result when subjected to χ^2 tests. The difference between the ultraviolet-irradiated and those with no exposure to ultraviolet was significant at a level of less than 0.05 and usually far less than 0.01 at each 50-day interval from 300 through 500 days. The hypothesis that mammary tumors appeared earlier among mice exposed to ultraviolet rays than those with no such exposure was tested further by a different application of the χ^2 method referred to by Snedecor (11) as "Test of independence in an R × C table." The technic may be applied when one or both of two attributes are recorded in more than two classes. This statistical treatment of the data indicated that, if the three groups of mice came from the same population, the probability of obtaining re-

TABLE 3

CUMULATIVE NUMBER OF MICE WITH MAMMARY TUMORS (Exp. 3)

Age (days)	Dark (12)	Light (15)	Light and U.V. (21)
250	0	2	3
300	0	3	7
350	2	3	13
400	6	8	16
450	8	12	19
500	10	12	20
550	10	12	21
600	12	12	
635		15	

TABLE 4

RELATIVE FREQUENCY OF MAMMARY TUMORS AT AGE 1 YEAR

	U.V. (per cent)	Dark (per cent)	Light (per cent)
Exp. 1	90	60	50
Exp. 2	74	57	46
Exp. 3	62	25	20

sults like those found in our experiments would be less than 0.05.

The earlier appearance of the mammary carcinomas among mice exposed to ultraviolet rays may have been due to a difference in the rate of tumor production or to a difference in latent interval, the rate of production being relatively constant. Blanding *et al.* (4) described a close correlation between tumor development and time expressed logarithmically among mice painted with some carcinogenic substances obtained from petroleum refineries. Similar treatment of the spontaneous tumor data from our experiments indicates a relationship of the order:

$$y = a + b \log x$$

in which y is the relative frequency of tumor production expressed as per cent and x the time of appearance of the mammary tumors expressed in days. Correlation coefficients of this relationship varied from 0.90 to 0.97. The slopes of the curves (b) for each group varied from 215 to 269. The differences were not statistically significant. The mean latent periods of tumor production and the standard errors of the means were:

Ultraviolet group	299 ± 10 days
Light group	391 ± 11 days
Dark group	369 ± 12 days

Differences among these means were not statistically significant.

SUMMARY

We are left, therefore, with good evidence that C3H mice exposed to ultraviolet rays developed spontaneous mammary cancers more rapidly than did those reared in either light or dark environments in which they received no ultraviolet irradiation. The differences are not due solely to a more rapid rate of tumor production or to a decreased latent interval, but to a combination of both.

ACKNOWLEDGMENTS

Grateful acknowledgment is made to three students, Miss Marilyn Wells, Miss Lois Schofield, and Mrs. Peter Cohen, for their assistance in carrying out these experiments.

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Announcements

Anna Fuller Memorial Prize

The second Anna Fuller Memorial Prize was presented on the occasion of the annual dinner of the American Association for Cancer Research, Inc., to Dr. Peyton Rous, Dr. F. Duran-Reynals, and Dr. R. E. Shope, on April 12, 1952. A certificate and a check were presented to each of these three investigators in recognition of their notable achievements in the demonstration of the role of viruses as causes of cancer: to Dr. Rous for his pioneer studies and demonstration of a virus as a cause of cancer in birds; to Dr. Shope for his work on the association of viruses with papillomas and fibromas in rabbits; to Dr. Duran-Reynals for his studies on the influence of the host on the responses to tumor-producing viruses and the probable mutation of such viruses. The recipients were nominated by Dr. John W. Cline, president of the American Medical Association, Dr. Alan M. Chesney, dean of Johns Hopkins University Medical School, and Dr. G. Parker Berry, dean of Harvard University Medical School, and the trustees of the fund.

Egbert C. Fuller, a businessman of New Haven and New York, created the Anna Fuller Fund in 1931 in memory of his wife. In his will he also provided for prizes to be awarded periodically in recognition of "real and outstanding contributions to knowledge on the subject of the cause, care, prevention, or cure of cancer." The second prize was awarded approximately 20 years after the fund became functional in March, 1932.

The first Anna Fuller Memorial Prize was awarded in 1939 to five scientists at the Research Institute of the Royal Cancer Hospital of London: Dr. E. L. Kennaway, Dr. James W. Cook, Dr. C. L. Hewitt, Dr. I. Hieger, and Dr. W. V. Mayneord. The award was made in recognition of the contributions of this group in the isolation and synthesis of cancer-producing hydrocarbons.

The Anna Fuller Fund, 205 Church Street, New Haven, Connecticut, was one of the first to be interested specifically in cancer.

Southwestern Section

American Association for Cancer Research

Two meetings of the Southwestern Section, American Association for Cancer Research, have been held during the year. The fall meeting was sponsored by Oklahoma Agricultural and Mechanical College in Stillwater, Oklahoma, on November 9, 1951. At this meeting, in addition to the regular program by the members, Dr. Moses Ashkenazy delivered (in conjunction with Dr. Herbert C. Allen) a guest lecture on "The Use of Radioactive Dyes and the Scintillation Counter in the Diagnosis of Brain Tumors." The by-laws presented to the membership by a special committee were adopted officially in modified form at this meeting.

The second meeting of the year was held in Dallas on March 14, 1952, at the Scottish Rite Hospital under the auspices of the Southwestern Medical School of the University of Texas. Guest speaker on that occasion was Dr. J. B. Trunnell, who spoke on "The Role of the Cancer Hospital in Research."

The officers of the society during the past year have been Dr. Stuart A. Wallace, president, Dr. J. M. Thuringer, vice-president, and Dr. W. J. Burdette, secretary-treasurer. Officers elected during the spring meeting for the coming year are Dr. Charles L. Spurr, president, Dr. M. R. Shetlar, vice-president, and Dr. W. J. Burdette, secretary-treasurer. The membership at the present time consists of seventeen full members and 81 associate members. Almost all the members of the national organization who reside in Arkansas, Louisiana, Oklahoma, or Texas are now active in the Southwestern Section.